



Attorney Docket No. 31200/69244
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: George Eustace JOANNOU

Conf. No.: 8120

Serial No.: 10/018,308

Group: 1625

Filed: January 24, 2002

Examiner: Amelia A. Owens

For: ISOFLAVONE METABOLITES

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SEP 26 2003
TECH CENTER 1600/69244

**PETITION TO REVIVE AN UNINTENTIONALLY
ABANDONED APPLICATION**

Mail Stop Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Under 37 C.F.R. §1.137, Applicant requests revival of the above-referenced application. The due date for paying the Issue Fee expired on September 16, 2003. Enclosed herewith are: (a) the Petition fee of \$650.00 and, (b) an Information Disclosure Statement (IDS) along with a Request for Continued Examination with filing fee. Please note that Applicant is a small entity.

The entire delay in filing the Request for Continued Examination until the filing of this Petition was unintentional.

A Terminal Disclaimer is not required.

The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Account No. 10-0435 (31200/69244).

Respectfully submitted,
BARNES & THORNBURG

Richard P. Krinsky
Registration No. 47,720
Tel. No. (202) 289-1313

RPK/sld

73632v1

09/25/2003 CCHAU1 00000004 10018308

01 FC:2453

650.00 DP



Attorney Docket No. 31200/69244
PATENT

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Serial No.: 10/018,308 Group: 1625
Filed: January 24, 2002 Examiner: Amelia A. Owens
For: ISOFLAVONE METABOLITES

REQUEST FOR CONTINUED EXAMINATION UNDER 37 C.F.R. § 1.114

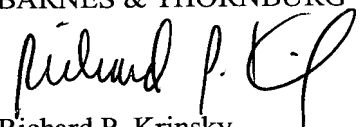
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant requests continued examination of the subject application and acceptance of the enclosed Information Disclosure Statement. A check for the required fee of \$375.00 is enclosed herewith. Applicant is a small entity.

It is respectfully requested that, if necessary to effect a timely response, this paper be considered as a Petition for an Extension of Time sufficient to effect a timely response and shortages in other fees be charged, or any overpayment in fees be credited, to the Account of Barnes & Thornburg, Deposit Account No. 10-0435 (31200/69244).

Respectfully submitted,
BARNES & THORNBURG


Richard P. Krinsky
Registration No. 47,720
(202) 289-1313

RPK/sld
Enclosures

09/25/2003 CCHAU1 00000004 10018308

02 FC:2801

375.00 00



Attorney Docket No. 31200/69244
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: George Eustace JOANNOU Conf. No.: 8120
Serial No.: 10/018,308 Group: 1625
Filed: January 24, 2002 Examiner: Amelia A. Owens
For: ISOFLAVONE METABOLITES

SUBMISSION UNDER 37 C.F.R. §1.56, §1.97 & §1.98
INFORMATION DISCLOSURE STATEMENT

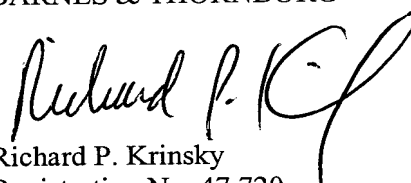
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

To comply with the duty of disclosure set forth in 37 C.F.R. §1.56, the prior art listed on the attached PTO-1449 is submitted herewith to the Examiner for consideration in connection with the examination of the above-identified application.

The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Account No. 10-0435 (31200/69244).

Respectfully submitted,
BARNES & THORNBURG


Richard P. Krinsky
Registration No. 47,720
(202) 289-1313

RPK/sld
Attachments

FORM PTO-1449

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTY DOCKET NO.

SERIAL NO.

31200/69244

10/018,308

LIST OF DOCUMENTS CITED BY APPLICANT

(Use several sheets if necessary)

APPLICANT

GEORGE EUSTACE JOANNOU

FILING DATE

JANUARY 24, 2002

GROUP

1625

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	5,352,384	10/1994	SHEN			
	5,569,459	10/1996	SHLYANKEVICH			
	5,424,331	06/1995	SHLYANKEVICH			
	5,523,087	06/1996	SHLYANKEVICH			
	4,264,509	04/1981	ZILLIKEN			
	4,234,577	11/1980	ZILLIKEN			

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
	EP 0129667	04/1984	EPO				
	EP 0135172	08/1984	EPO				
	DE 4432947	03/1996	GERMANY				
	WO 98/21946	11/1997	WIPO				
	WO 97/06273	08/1996	WIPO				
	WO 94/23716	04/1994	WIPO				
	WO 00/64438	04/2000	WIPO				
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EXAMINER:

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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

FORM PTO-1449

U.S. DEPARTMENT OF COMMERCE
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SERIAL NO.

31200/69244

10/018,308

LIST OF DOCUMENTS CITED BY APPLICANT

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GEORGE EUSTACE JOANNOU

FILING DATE

JANUARY 24, 2002

GROUP

1625

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(1992) *Biochemical Pharmacology*, Vol. 44, No. 1, pp. 157-162
- M. Weidenborner et al., *Control of Storage Fungi of the Genus Aspergillus on Legumes with Flavonoids and Isoflavonoids*,
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U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTY DOCKET NO.

SERIAL NO.

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APPLICANT

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LIST OF DOCUMENTS CITED BY APPLICANT

(Use several sheets if necessary)

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		K. Mackenbrock et al., <i>3'-Hydroxylation of 4'-Methoxyisoflavones by Fusarium oxysporum f. lycopersici</i> , (1983) Z. Naturforsch 38c, pp. 708-710
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		Answers 1-19 CA Copyright 2000 ACS

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(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 84104618.8

(51) Int. Cl.⁴: A 61 K 31/35

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(30) Priority: 26.04.83 JP 74575/83

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02.01.85 Bulletin 85/1

(54) Designated Contracting States:
BE CH DE FR GB LI NL SE

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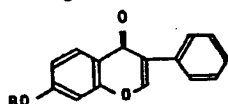
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(54) Compounds and pharmaceutical compositions for treatment of hypoovarianism.

(57) A compound of the general formula



[wherein R is a hydrogen atom or a lower alkyl group] is effective for treatment of hypoovarianism.

EP 0 129 667 A1

TITLE MODIFIED

see front page

- 1 -

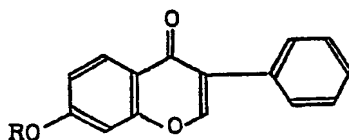
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Method for Treatment of Hypoovarianism

This invention relates to a therapeutic means for treatment of hypoovarianism.

More particularly, this invention relates to a medicament containing a compound of the general formula

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[wherein R is a hydrogen atom or a lower alkyl group]

for treatment of hypoovarianism. Hypoovarianism such as climacteric disturbances, infertility, etc. is caused by a decreased secretion of estrogen [hereinafter referred to as E] due to hypofunction of the ovary.

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Climacteric disturbances are the syndrome which develop in females in climacterium (approximately at the ages of 45 to 60) as a decreased secretion of E results in changes in various metabolisms of the body which, in turn, affect the diencephalon which is the autonomic nerve center. Manifestations of climacteric disturbances are diverse and include angioneurotic disorder-like symptoms such as heat sensation, feeling of cold, hot flash, palpitation, etc., psychoneurotic disorder-like symptoms such as headache, dizziness, insomnia, etc.; perception disorder symptoms such as numbness, hyperesthesia, hypesthesia, etc.; locomotorial disorder symptoms such as lumbago, shoulder stiffness, arthralgia, etc.; cutaneous

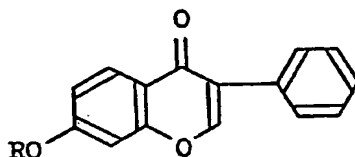
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secretory disturbance symptoms such as perspiration, dry mouth, etc.; obesity, emaciation, climacteric diabetes, senile vaginitis, pruritus cutaneus, fatigue, arteriosclerosis, cardiac disorder and so on. For the treatment of these manifestations of climacteric disturbance, therapies using a synthetic E drug or an androgen drug or both of them so as to correct for the decrease in secretion of endogenous ovarian hormone proved successful and were used as therapies of choice against climacteric disturbances. In recent years, however, these therapies have not been used as often as in the past because they present the risk of metrorrhagia and carcinogenicity. As other therapies, minor tranquilizers, antidepressants, autonomic blocking agents, peripheral circulation improving agents, chinese medicines, etc. have been administered but their efficacies are not satisfactory as are desired.

Infertility, which is said to be present in 5 to 10 percent of all the females of fertile ages (approximately up to the early forties), is caused by various causes and includes such cases due to insufficient E secretion as ovarian aplasia, ovulation disorder, uterine aplasia, nidation disorder, early abortion, etc. Against these types of infertility, synthetic E drugs and progesterones have been mainly employed. However, these drugs have the disadvantage that responses vary a great deal according to individuals.

The present inventors found that the compound of general formula (I)



(I)

[hereinafter there may be occasionally referred to as the Compound (I) and wherein R is a hydrogen atom or a lower alkyl group] does not independently have E activity but does potentiate E activity and demonstrated that the compound potentiates E activity of ovarian origin which has been depressed in patients with climacteric disturbances or infertility, thus being useful for the treatment of these diseases.

Thus, the principal object of this invention is to provide a method for treatment of hypoovarianism through the mechanisms of potentiating the activity of E produced endogenously in the human ovary or administered exogenously, which comprises administering to the female the compound of general formula (I). Another object of this invention is to provide a pharmaceutical composition comprising the compound of general formula (I), which is usable in the above-mentioned method.

Referring, now, to the above general formula (I), the lower alkyl group R may be a straight-chain group or a branched-chain group, and may for example be methyl, ethyl, propyl, butyl or pentyl. The compound of general formula (I) can be produced, for example by cyclizing a 2-hydroxy-4-RO-substituted phenyl benzyl ketone in the conventional manner and the compound (I) wherein R is a lower alkyl group can also be produced by alkylating a 7-hydroxy-isoflavone.

The compound of general formula (I) which is employed in accordance with this invention is invariably a white to pale yellowish brown crystalline compound which is freely soluble in dimethylformamide and chloroform, soluble in ethanol and acetone, and practically insoluble in water.

As will be apparent from acute toxicity test shown hereinafter, the compound of general formula (I) did not cause death nor toxic symptoms attributable thereto when its technically feasible maximum dose (5,000 to 10,000 mg/kg) was administered orally or subcutaneously to mice and rats. Thus, the compound of general formula (I) is only sparingly toxic.

Methods for the preparation of compound (I) are described in the prior art and some species of compound (I) and related isoflavones are known to be useful as agents for increasing capillary strength (French Pharmaceutical Patent No. 1065), therapeutic drugs for vascular disorder, inflammation and P-hypovitaminosis conditions (United States Patent No. 3352754), body weight increasing agents (United States Patent Nos. 3864362, 4166862 and 4117149), agents for increasing muscle power, anticatabolic agents and antasthenics (United States Patent No. 3864362), therapeutic drugs for osteoporosis of immobilisation (United States Patent No. 3907830), and therapeutic drugs for myocardial or pulmonary insufficiency (Japanese Toku-Kai-Sho 53-133635), etc. However, nothing is known about the use of the compound as a therapeutic drug for hypoovarianism.

The dosage of the compound of general formula (I) according to this invention for humans is generally about 5 to 10 mg/kg/day for oral administration, and about 200 to 600 mg can be orally taken daily, once a day or, if necessary, in 2 to 3 divided doses. The compound (I) is preferably formulated into such dosage forms as tablets, capsules, etc. by the established pharmaceutical procedure. Such tablets and capsules can be prepared using suitable vehicle such as lactose, starch, etc., binders such as hydroxypropylcellulose, and lubricants such as magnesium stearate. The tablets may be sugar-coated, if necessary.

The following experimental examples show the activity of the compound of general formula (I) to potentiate E activity. In these examples, the species of the compound of general formula (I) used were 7-hydroxy-
 5 isoflavone [hereinafter referred to as Compound A] and 7-isopropoxyloxy-isoflavone [briefly, Compound B].

Test Example 1

E activity-potentiating effect of 7-hydroxy-isoflavone
 10 in young oophorectomized rats

Sprague-Dawley rats, 33 days of age and 11 days after oophorectomy for elimination of endogenous E activity, were assigned to groups of 5 to 7 individuals and treated subcutaneously with solutions of esterone
 15 in sesame oil or orally with suspensions of Compound A in 1% hydroxypropylcellulose solution, either independently or in combination, for 3 days. On the 4th day, each animal was autopsied and its uterine wet weight was recorded. As shown in Table 1, Compound A
 20 at 50 and 100 mg/kg did not cause an increase of uterine weight but potentiated the uterine weight increasing action of estrone.

Table 1

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Daily dose of estrone (μg/kg)	Daily dose of Compound A (mg/kg)	No. of animals	Uterine wet weight (mg±S.D.)
0	0	5	37.4 ± 0.6
0	50	7	35.3 ± 1.7
0	100	7	35.9 ± 1.0
1.0	0	6	52.6 ± 2.3
1.0	50	6	60.8 ± 4.1
1.0	100	6	70.6 ± 2.7*

*: Significant as compared with estrone 1.0 µg/kg group (P<0.01).

Test Example 2

5 E activity-potentiating effect of 7-isopropoxy-isoflavone in young oophorectomized rats

10 Sprague-Dawley rats, 33 days of age and 11 days after oophorectomy for elimination of endogenous E activity, were assigned to groups of 5 to 7 individuals and treated subcutaneously with solutions of esterone in sesame oil or orally with suspensions of Compound B in 1% hydroxypropylcellulose solution, either independently or in combination, for 3 days. On the 15 4th day, each animal was autopsied and its uterine wet weight was recorded. As shown in Table 2, Compound B at 50 and 200 mg/kg did not cause an increase of uterine weight but potentiated the uterine weight increasing action of estrone.

20 Table 2

	Daily dose of estrone (µg/kg)	Daily dose of Compound B (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
25	0	0	7	29.2 ± 0.9
	0	50	7	32.2 ± 1.3
	0	200	7	34.7 ± 1.2
	0.5	0	7	36.3 ± 1.9
	0.5	50	7	39.6 ± 2.4
30	0.5	200	7	51.3 ± 5.3*
	1.0	0	7	51.9 ± 4.2
	1.0	50	7	58.2 ± 2.6
	1.0	200	7	71.5 ± 7.4 ⁺

35 *: Significant as compared with estrone 0.5 µg/kg group (P < 0.05).

+: Significant as compared with estrone 1.0 µg/kg group (P < 0.05)

Acute toxicity

Five-week-old ICR mice and 5-week-old Spraque-Dawley rats were used in groups of 10 males and 10 females, and suspensions of compound A or B in olive oil were administered orally [2,500, 5,000 and 10,000 mg/kg of each compound] or subcutaneously (1,250, 2,500 and 5,000 mg/kg). The animals were kept under observation for 14 days. None of the groups showed deaths nor toxic symptoms which might be attributable to compound A or B, with the result that LD₅₀ could not be calculated.

The following preparation examples are given to illustrate the invention in further detail only and not to limit the scope of the invention thereto.

Preparation Example

1. Tablets

20	I) 7-Isopropoxy-isoflavone	200 g
	II) Lactose	15 g
	III) Starch	44 g
	IV) ECG-500	10 g
	V) Magnesium stearate	1 g

25 The above components I) through V) were admixed to prepare 1000 uncoated tablets with a diameter of 8.5 mm.

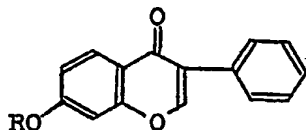
2. Capsules

	I) 7-Hydroxy-isoflavone	200 g
30	II) Lactose	40 g
	III) Starch	50 g
	IV) Hydroxypropylcellulose	7 g
	V) Magnesium stearate	3 g

35 The above components I) through V) were admixed and filled into 1000 No. 1 capsules.

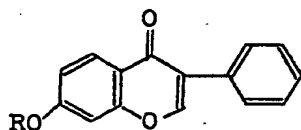
What is claimed is:

1. A compound of the general formula



[wherein R is a hydrogen atom or a lower alkyl group]
for use in treatment of hypoovarianism.

2. A pharmaceutical composition for treatment of hypo-ovarianism, which contains an effective amount of a compound of the general formula



[wherein R is a hydrogen atom or a lower alkyl group]
and a pharmaceutical acceptable carrier, vehicle, lubricant or diluent therefor.

3. A pharmaceutical composition according to claim 2, which is in the form of tablet, capsule, granule, fine granule, powder or syrup.
4. A pharmaceutical composition according to claim 2, wherein the hypoovarianism is climacteric disturbances or infertility.



European Patent
Office

EUROPEAN SEARCH REPORT

0129667

Application number

EP 84104618.8

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
D,X	FR - M - 1 065 (LABORATORIES LAROCHE-NAVARRON) * Claims 1,2,4,6 *	1-4	A 61 K 31/35
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D,X	US - A - 3 352 754 (J.M. GAZAVE) * Column 1, lines 50-67; column 4, lines 32-49; column 5, lines 8-16 *	1-4	
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X	DE - A - 2 166 458 (CHINOIN GYOGYSZER ES VEGYESZETI TERMEKEK GYARA RT) * Page 5, line 26 - page 7, line 19 *	1-4	
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D,X	US - A - 3 907 830 (L. FEUER et al.) * Column 7, line 58 - column 8, line 20; column 11, lines 24-52 *	1-4	TECHNICAL FIELDS SEARCHED (Int. Cl. 7) A 61 K 31/00 A 23 K 1/00
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D,X	US - A - 3 864 362 (L. FEUER et al.) * Column 1, line 46 - column 2, line 45 *	1-4	

The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 11-07-1984	Examiner MAZZUCCO
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p>			

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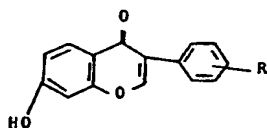
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54 Method for treatment of osteoporosis.

57 A compound of the formula



wherein R is a hydrogen atom or a hydroxy group is effective for prevention or treatment of osteoporosis.

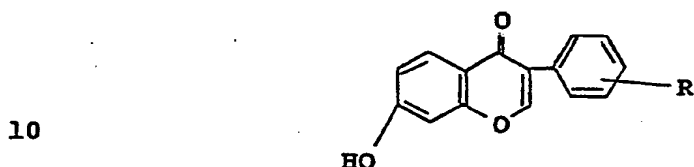
EP 0 135 172 A2

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Method for treatment of osteoporosis

This invention relates to a therapeutic means for treatment of osteoporosis.

More particularly, this invention relates to a medicament
5 for prevention or treatment of osteoporosis, which contains
a compound of the formula



wherein R is a hydrogen atom or a hydroxy group.

Osteoporosis is a disease condition or illness which
15 occurs frequently in postmenopausal females, particularly
those in their sixties, and wherein the quantitative loss
of bones progresses beyond a certain limit to thereby present
some symptoms or risk manifestations. Among its main clinical
manifestations are kyphosis, low back pain, and fractures
20 of femoral neck, lower end of the radius, ribs, upper end
of the humerus, etc. While the causative factors are variegated,
including endocrine disorder and nutritional disorder, apparently
the most important cause is a decreased secretion of estrogen
due to hypoovarianism in females during the postmenopausal
25 period. Therefore, of all the therapeutic agents for osteoporosis,
the theoretically most effective drugs are estrogen preparations.

However, the estrogens so far available are so strong in effect as to cause side effects such as genital bleeding, mastodynia, hepatic disorder, etc. and, for this reason, have not been used recently on as many occasions as in the past. There are other types of therapeutic agents such as calcitonin, vitamin D and calcium preparations, which however are disadvantageous in that they are either only indefinitely effective or ineffective when administered by the oral route.

10 The present inventors have found that the compound of the formula (I) exhibits a milder estrogen activity than the conventional estrogens in the oral regimen and does not cause side effects which are produced by these known drugs but cures osteoporosis by stimulating secretion of
15 calcitonin from the thyroid.

 The compounds of the formula (I) which are employed in accordance with the present invention are invariably crystalline compounds which are white to pale yellowish brown in color, and are freely soluble in dimethylformamide
20 and chloroform, soluble in ethanol and acetone and practically insoluble in water. When R in the formula (I) is a hydroxy group, it may be present in any position of the phenyl ring.

 These compounds can be produced, for example, by cyclizing a 2,4-dihydroxy-phenyl (with or without a hydroxy group
25 on the benzene ring) benzyl ketone to a benzopyran compound, and some of these compounds are known to have capillary vessel stabilizing activity (French Pharmaceutical Patent No. 1065), therapeutic effective for vascular disorders, inflammatory and vitamin-P deficiency disorders (United
30 States Patent No. 3,352,754) or anticonvulsant activity (Japanese Patent Publication No. 32074/1972), but it has not been known that any of the compounds is useful for the treatment of osteoporosis.

 As will be apparent from Test Example 5 which appears
35 hereinafter, all of the compounds of the formula (I) are

sparingly toxic. Thus, in the studies in which the compounds were administered orally or subcutaneously to mice or rats at the technically feasible highest doses (5,000 to 10,000 mg/kg), there occurred no death nor toxic symptoms attributable to the compounds.

On the other hand, Test Examples 1 and 2 presented hereinafter show that 7-hydroxy-isoflavone [hereinafter referred to briefly as compound (I)] and 7,4'-dihydroxy-isoflavone [briefly, compound (II)], which are representative species of the compound represented by the formula (I), have mild estrogenic activity which is suited for the treatment of osteoporosis.

Test Example 1

Estrogenic activity of 7-hydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 6 to 7 animals. Compound (I) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally for 3 days, while as a representative example of the conventional estrogen drug, estrone was dissolved in sesame oil and administered subcutaneously for 3 days. On the fourth day, each animal was autopsied and its uterine wet weight was recorded. As shown in Table 1, compound (I) at the daily dose levels of 200 mg/kg and 400 mg/kg produced uterine weight increasing effect with a dose-response curve of moderate gradient. In contrast, estrone showed uterine weight increasing effect with a dose-response curve of steep gradient.

Table 1

Compound	Daily dose (mg/kg)	No. of animals	Uterine wet weight (mg \pm S.D.)
Compound (I)	0 (control group)	7	35.0 \pm 1.0
	6.25	7	32.8 \pm 1.1
	12.5	7	33.4 \pm 0.9
	25	7	35.1 \pm 0.8
	50	7	35.3 \pm 1.7
	100	7	35.9 \pm 1.0
	200	7	57.9 \pm 1.0*
	400	6	70.4 \pm 6.7*
Estrone	0.0025	7	101.7 \pm 4.6*
	0.005	7	159.8 \pm 9.4*
	0.01	7	223.3 \pm 12.5*

*: Significant as compared with control group ($P < 0.01$)

Test Example 2

Estrogenic activity of 7,4'-dihydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 7 animals. Compound (II) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally. As shown in Table 2, compound (II) at the dose level of 400 mg/kg showed mild uterine weight increasing activity.

Table 2

Daily dose of compound (II) (mg/kg)	No. of animals	Uterine wet weight (mg \pm S.D.)
0 (control group)	7	31.1 \pm 1.1
6.25	7	33.2 \pm 0.8
25	7	32.8 \pm 1.0
100	7	35.3 \pm 1.3
400	7	62.3 \pm 6.0*

*: Significant as compared with control group. ($P < 0.01$)

The following Test Examples 3 and 4 show that the compounds of this invention have bone resorption-inhibiting activity which is effective for the treatment of osteoporosis.

Test Example 3

Bone resorption inhibiting activity of 7-hydroxy-isoflavone and 7,4'-dihydroxy-isoflavone in rat fetal long bone culture.

Determination of bone resorption was performed by the method of Raisz [J. Clin. Invest. 44, 103-116 (1965)]. Thus, a Sprague-Dawley rat on the 19th day of pregnancy was subcutaneously injected with 50 μ Ci of ^{45}Ca (isotope of calcium, CaCl_2 solution), and was laparotomized on the following day. The embryos were aseptically taken out, the forelimbs (radius and ulna) were cut off from the trunk under a binocular dissecting microscope, and the connective tissue and cartilage were removed as much as possible to prepare bone samples. Each bone sample was preincubated at 37°C for 24 hours in 0.6 ml of the medium containing 2 mg/ml of bovine serum albumin in BGJ_b medium (Fitton-Jackson modification) [GIBCO Laboratories, Grand Island, NY 14072 U.S.A.]. Then, the sample was further incubated for 3 days in the same medium as above in which 10 $\mu\text{g/ml}$ or 25 $\mu\text{g/ml}$ of compound (I) or 10 $\mu\text{g/ml}$ of compound (II) had been incorporated. Then, the radioactivity of ^{45}Ca in the medium and that of ^{45}Ca in the bone were measured and the percentage (%) of

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^{45}Ca released from the bone into the medium was calculated by the following formula.

Percentage (%) of ^{45}Ca released from bone into medium

$$5 = \frac{\text{Count of } ^{45}\text{Ca in medium}}{\text{Count of } ^{45}\text{Ca in medium} + \text{Count of } ^{45}\text{Ca in bone}} \times 100$$

As control, the bones of the embryos from the same litter were similarly incubated in the absence of compound (I) or (II) for 3 days. The mean \pm standard deviation for the six bones per group are shown in Table 3. It is apparent that compounds (I) and (II) suppressed bone resorption.

Table 3

	Concentration of compound	^{45}Ca (%) released	
15	Control group	0	20.6 \pm 3.8 19.9 \pm 5.0
	Test group 1	Compound (I) 10 $\mu\text{g/ml}$	16.5 \pm 2.5*
20	Test group 2	Compound (I) 25 $\mu\text{g/ml}$	13.5 \pm 2.5*
	Test group 3	Compound (II) 10 $\mu\text{g/ml}$	15.9 \pm 1.3**

25 * : A significant difference from the control group ($P < 0.001$)

** : A significant difference from the control group ($P < 0.002$)

Test Example 4

Inhibiting activity of 7,4'-dihydroxy-isoflavone to the bone resorption potentiating action of parathyroid hormone in rat fetal long bone culture.

30 The bone samples prepared in the same manner as Test Example 3 were pre-incubated for 24 hours in the same medium as that prepared in Test Example 3 which contains bovine serum albumin in BGJ_b medium (Fitton-Jackson modification).
35 Then, in the concomitant presence of PTH (parathyroid hormone, a bone resorption stimulant) and compound (II), the samples

were further incubated for 3 days and the percentage of ^{45}Ca released into the medium was calculated by means of the same formula as that in Test Example 3. The results are shown in Table 4. As control experiments, the same determination was made for a control group using the medium supplemented with PTH alone. It is apparent from Table 4 that compound (II) suppressed PTH-stimulated bone resorption.

Table 4

	<u>Concentration of compound (II)</u>	<u>^{45}Ca (%) released</u>
Control group	0	30.8 \pm 4.3
Test group	10 $\mu\text{g/ml}$	23.5 \pm 3.4*

*: A significant difference from the control group ($P < 0.01$)

Test Example 5

Acute toxicity

Five-week-old ICR mice and 5-week-old

Sprague-Dawley rats were respectively used in groups of 10 males and 10 females, and suspensions of compound (I) or compound (II) in olive oil were administered orally [2,500, 5,000 and 10,000 mg/kg of each compound] or subcutaneously [1,250, 2,500 and 5,000 mg/kg]. The animals were kept under observation for 14 days. None of the groups showed deaths nor toxic symptoms attributable to compound (I) or (II) and, therefore, LD_{50} values could not be calculated.

The daily dosage of the compound of the formula (I) according to this invention for human beings is generally about 1 to 50 mg/kg and preferably about 5 to 20 mg/kg for oral administration, and about 200 to 600 mg can be orally taken daily, once a day or, if necessary, in 2 to 3 divided doses. The compounds are preferably formulated into such dosage forms as tablets, capsules, etc. by the established pharmaceutical procedure. Such tablets and capsules can

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be prepared using suitable excipients such as lactose, starch, etc., binders such as hydroxypropylcellulose, and lubricants such as magnesium stearate. The tablets may be sugar-coated, if necessary.

5 The following preparation examples are given to illustrate the invention in further detail and should not be construed as limiting the scope of the invention.

Example 1 Tablets

	I) 7-Hydroxy-isoflavone	200 g
10	II) Lactose	15 g
	III) Starch	44 g
	IV) Carboxymethylcellulose	10 g
	V) Magnesium stearate	1 g

15 The above components I) through V) were admixed to prepare 1000 uncoated tablets with a diameter of 8.5 mm.

Example 2 Capsules

	I) 7,4'-Dihydroxy-isoflavone	200 g
	II) Lactose	40 g
	III) Starch	50 g
20	IV) Hydroxypropylcellulose	7 g
	V) Magnesium stearate	3 g

 The above components I) through V) were admixed and filled into 1000 No. 1 capsules.

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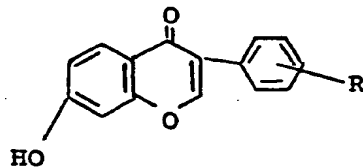
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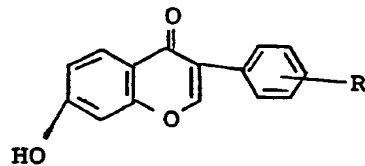
What is claimed is:

1. A compound of the formula



wherein R is a hydrogen atom or a hydroxy group for use in prevention or treatment of osteoporosis.

2. A pharmaceutical composition for prevention or treatment of osteoporosis, which contains an effective amount of a compound of the formula



wherein R is a hydrogen atom or a hydroxy group and a pharmaceutical acceptable carrier, vehicle, lubricant or diluent therefor.

3. A pharmaceutical composition according to claim 2, which is in the form of tablet, capsule, granule, fine granule, powder or syrup.

4. A pharmaceutical composition according to claim 2, wherein the osteoporosis is that caused by decreasing secretion of estrogen due to hypoovarianism.



①⑨ BUNDESREPUBLIK
DEUTSCHLAND



DEUTSCHES
PATENTAMT

①⑫ **Offenl gungsschrift**
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Prüfungsantrag gem. § 44 PatG ist gestellt

⑤④ Mittel zur Behandlung der Haut

⑤⑦ Die vorliegende Erfindung betrifft ein neues Mittel zur
Behandlung der Haut, das als Wirkstoff Isoflavon enthält.

DE 44 32 947 A 1

Beschreibung

Die vorliegende Erfindung betrifft ein neuartiges Mittel zur Behandlung der Haut, insbesondere zur kosmetischen oder medizinischen Anwendung.

Zur Pflege und Behandlung der Haut werden auf dem Markt zahlreiche Produkte angeboten. Es handelt sich hierbei im wesentlichen um Lotionen, Milche, Cremes und Pasten.

Die angebotenen Reinigungs-lotionen, -milche, -cremes und -pasten basieren zumeist auf Öl-/Wasseremulsionen mit relativ geringen Gehalten an Fettkomponenten mit reinigenden und pflegenden Zusätzen. Die angebotene breite Palette verschiedener Hautreinigungsprodukte variiert in Zusammensetzung und Gehalt an diversen Wirkstoffen, abgestimmt auf die verschiedenen Hauttypen und spezielle Behandlungsziele.

Die der Reinigung folgende Hautpflege hat zwei wesentliche Ziele: Zum einen soll sie der Haut die bei der Wäsche unkontrolliert entzogenen Inhaltsstoffe wie Hornzellen, Hautfettlipide, Säurebildner und Wasser zurückführen in den natürlichen Gleichgewichtszustand. Zum anderen sollen sie den natürlichen Alterungsprozeß der Haut sowie den möglichen Schädigungen durch Witterungs- und Umwelteinflüsse weitgehend entgegenwirken. Präparate zur Hautpflege und zum Hautschutz werden in großer Zahl und in vielen Zubereitungsformen angeboten. Die wichtigsten sind Hautcremes, -lotionen, -öle und -gele. Basis der Cremes und Lotionen sind Emulsionen Öl in Wasser oder Wasser in Öl-Form. Die Hauptbestandteile der Ölbzw. Fett- oder Lipid-Phase sind Fettalkohole, Fettsäuren, Fettsäureester, Wachse, Vaseline, Paraffine sowie weitere Fett- und Ölkomponenten hauptsächlich natürlichen Ursprungs. In der wäßrigen Phase sind neben Wasser hauptsächlich feuchtigkeitsregulierende und feuchtigkeitsbewahrende Substanzen als wesentlicher Hautpflege-Wirkstoff enthalten, ferner konsistenz- bzw. viskositätsregulierende Mittel. Weitere Zusätze wie Konservierungsmittel, Antioxidanzien, Komplexbildner, Parfüm, Öle, Färbemittel sowie spezielle Wirkstoffe werden je nach ihrer Löslichkeit und ihren Stabilitätseigenschaften einer der beiden vorgenannten Phasen beigegeben.

Hautöle gehören zu den ältesten Produkten der Hautpflege und werden heute noch verwendet. Basis sind nicht trocknende Pflanzenöle, wie Mandelöl oder Olivenöl mit Zusätzen natürlicher Vitaminöle wie Weizenkeimöl oder Avokadoöl sowie öligen Pflanzenextrakten, z. B. Johanniskraut, Kamille und ähnlichen. Der Zusatz von Antioxidanzien gegen Ranzigkeit ist unerläßlich. Gewünschte Duftnoten werden durch Parfüm oder etherische Öle erzielt. Ein Zusatz von Paraffinöl oder flüssigen Fettsäureestern dient zur Optimierung der Anwendungseigenschaften.

Hautgele sind halbfeste transparente Produkte, die durch entsprechende Gelbildner stabilisiert werden. Man unterscheidet Oleogele, Hydrogele und Öl/Wasser-Gele. Die Typenauswahl richtet sich nach dem gewünschten Anwendungszweck. Die Öl/Wasser-Gele enthalten hohe Emulgator-Anteile und weisen gegenüber Emulsionen gewisse Vorteile auf.

Zu den kosmetischen Mitteln gehören auch die Haarbehandlungsmittel. Denn die Haarpflege umfaßt die Reinigung von Kopfhaut und Kopfhaar gleichermaßen. Demzufolge enthalten die Haarpflegeprodukte hautpflegenden Wirkstoffe.

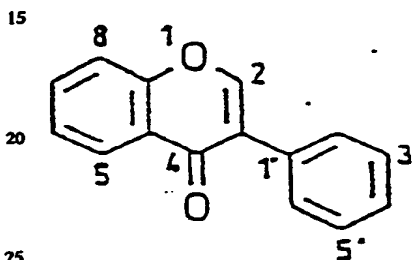
Nachteilig bei den genannten kosmetischen Mitteln

ist die Begrenzung der Wirksamkeit.

Die vorliegende Erfindung hat sich nunmehr die Aufgabe gestellt, ein Mittel zur Behandlung der Haut zur Verfügung zu stellen, das für die genannten Anwendungsgebiete einen wirksamen Wirkstoffkomplex aufweist.

Diese Aufgabe wird dadurch gelöst, daß das Mittel als Wirkstoff Isoflavon enthält.

Bei dem erfindungsgemäßen Isoflavon handelt es sich um die meist zu den Flavonoiden gezählte, gelegentlich auch als Isoflavonoide bezeichnete Gruppe von Stoffen, die sich von 3-Phenyl-4-H-1-benzopyran-4-on der folgenden Strukturformel ableiten:



Bevorzugt werden erfindungsgemäß 4-Hydroxy-7-Glucose-Isoflavon (Daidzin), 5,7,4'-Trihydroxy-Isoflavon (Genistein), 5,4'-Dihydroxy-7-Glucose-Isoflavon (Genistin), 7,4'-Dihydroxy-Isoflavon (Daidzein), 5,7-Dihydroxy-4'-methoxy-Isoflavon (Biochanin A), 3',4',5,7-Tetrahydroxyisoflavon (Orobol), 3',4',5-Trihydroxy-7-methoxyisoflavon (Santal), 3',5,7-Trihydroxy-4'-methoxyisoflavon (Pratensein), 3',5,7-Trihydroxy-4',5',6-trimethoxyisoflavon (Iridin).

Daneben kommen auch die Abkömmlinge des Isoflavons in Betracht, z. B. die in C-2 und C-3-Stellung hydrierten Isoflavone. Ebenso können sämtliche Isoflavonderivate erfindungsgemäß zum Einsatz kommen. Hierzu zählen vor allem Glykoside, Ether, Ester und von den Isoflavonen abgeleitete Säuren.

Die erfindungsgemäßen Wirkstoffe werden vorzugsweise in Konzentrationen von 0,1 bis 0,005 Gew.-% eingesetzt. Hierbei können in dem Mittel zusätzlich Sterol enthalten sein.

Aus der jüngsten Literatur ist die medizinische Anwendung von Isoflavonoiden zwar bekannt. Doch handelt es sich hierbei im wesentlichen um Forschungen auf dem Gebiet der Krebsvorsorge. Diese basierten auf der Erkenntnis, daß es in der Tumorrate bei Europäern und Asiaten, insbesondere im Bereich von Prostataerkrankungen und Brustkrebs signifikante Unterschiede gibt. Hierbei war man darauf gestoßen, daß der hohe Anteil an Sojaprodukten in der asiatischen Ernährung eine Ursache zu sein scheint. Im Rahmen der weiteren Arbeiten wurden sodann die Isoflavonoide als Wirkstoffe aus den Sojaprodukten isoliert und auf ihre krebshemmenden Wirkungen hin untersucht.

Ein Hinweis auf die erfindungsgemäß überraschend breiten Anwendungsbereiche findet sich jedoch in diesen Forschungsarbeiten nicht. Ebenso war es bei den bisherigen Kosmetika und medizinischen Mitteln zur Behandlung der Haut nicht bekannt, die erfindungsgemäß breite Wirkungspalette unter Einsatz nur eines einzigen Wirkstoffes zu erreichen. Denn das neue Mittel eignet sich zur Minderung der Gefäßneubildung (Couperose), der Behandlung erweiterter Venen (Besenreiser), Akne, Fetthaut, ergrauten Haaren und Pigment-

flecken. Insgesamt wirkt das neue Mittel antiproliferativ bei Melanomen, Alopecie, Akne und im Haarbulbus. Darüber hinaus kann mit dem erfindungsgemäßen Mittel der Hautalterung vorgebeugt werden, da Untersuchungen ergeben haben, daß es auch als Radikalfänger wirkt.

Die erfindungsgemäß eingesetzten Isoflavone lassen sich aus Zuckerfraktionen von Pflanzen (Obst, Getreide, Gemüse, Bohnen, Kirschen, Weizen, Hafer, Soja, Erbsen, Linsen, Möhren, Pfirsiche, Kohl, Peranja-Wurzel) oder Mikroorganismen (Pseudomonas) gewinnen. Hierbei können die Isoflavone in reiner Form isoliert werden und direkt als Wirkstoff in das Mittel eingegeben werden.

Ebenso ist es aber auch möglich, Isoflavone synthetisch herzustellen und für die erfindungsgemäßen Zwecke einzusetzen.

Schließlich können auch ethanolische, wäßrige Extrakte aus Pflanzen, Mikroorganismen oder synthetischen Gemischen zum Einsatz kommen. Hierbei liegt der Ethanolgehalt vorzugsweise zwischen 100 und 60 Gew.-%.

Auch ist es denkbar, Hydrolyseprodukte aus Pflanzen oder Mikroorganismen bzw. deren Zuckerfraktionen oder den Extrakten der Pflanzen, Mikroorganismen und Synthesegemische herzustellen.

Im folgenden wird die Erfindung unter Bezugnahme auf die Beispiele näher beschrieben.

Beispiel 1

Um die Minderung von Blutgefäß-Neubildungen nachzuweisen, wurden die Rezepturen 2 und 3 täglich dreimal auf die entsprechenden Zonen aufgetragen. Ebenso wurde der Einsatz als Radikalfänger und Pigmentflecken-Minderer nachgewiesen. Die Kontrolle nach 8 Wochen ergab jeweils eine leichte Minderung der Blutgefäß-Neubildungen und der Pigmentflecken.

Beispiel 2

Die Proliferationsrate-Senkung wurde im Falle von Sebumproduktion Haarwuchs und Hyperkeratinisierung bei Akne geprüft. Dazu wurden die Rezepturen 1 und 2 eingesetzt. Bei der Anwendung der Rezeptur 1 wurde täglich mehrmals auf die entsprechenden Areale mit Hilfe eines handelsüblichen Pumpspray-Fläschchens appliziert; im Falle der Rezeptur 2 wurde dünn auf die entsprechenden Hautareale aufgetragen.

Als Ergebnis wurde folgendes festgestellt: Minderung des Aknegradings nach 15 Tagen und Minderung der Fetthaut nach 4 Wochen. Die Haarwuchsrate konnte erst nach 2 Monaten kontrolliert werden (Messung des Wuchses in 2 Monaten) und zeigte eine minimale Erniedrigung.

Beispiel 3

Die Grauhaar-Minderung und haarwuchsregulierende Wirkung bei androgenetischer Alopecie wurden mit dem Spray gemäß Rezeptur 5 erprobt. Es wurde nach Monaten eine geringe Ausfallquote bemerkt (Verlängerung der Anagenphase), was den Einfluß auf der Ebene der hormonellen Regulation ohne Hormoneinsatz beweist.

Rezept 1: Spray, Ampullen-Flüssigkeit

Wasser dem.	7 Gew.-%
Ethanol denat.	83 Gew.-%
Sojaextrakt G	10 Gew.-%

Rezept 2: Isoflavon-Gel

Phase 1:	
Eumulgin B1	3 Gew.-%
Cetiol 868	10 Gew.-%
Methylparaben	0,15 Gew.-%
Propylparaben	0,10 Gew.-%
Sojaextrakt G	10,0 Gew.-%
Phase 2:	
Wasser	73 Gew.-%
Phase 3:	
Sepigel 305	3,5 Gew.-%
Phase 4:	
Kathon CG	0,05 Gew.-%

Herstellungsvorschrift

Phase 1 wird bei ca. 60 Grad aufgeschmolzen. Phase 2 wird auf ca. 60 Grad erwärmt. Danach werden beide Phasen zusammengegeben und verrührt. Anschließend wird Phase 3 zugegeben und bis zum Gelzustand blasenfrei verrührt. Schließlich wird nach Abkühlung auf ca. 30 Grad Phase 4 zugesetzt.

Rezept 3: Isoflavon-Lotion

Phase 1:	
Eumulgin B2	3,5 Gew.-%
Cutina MD	5 Gew.-%
Cetiol 868	7 Gew.-%
Jobaöl	3 Gew.-%
Methylparaben	0,15 Gew.-%
Propylparaben	0,10 Gew.-%
Sojaextrakt G	10 Gew.-%
Phase 2:	
Wasser	70,5 Gew.-%
Carbapol 934	0,30 Gew.-%
Phase 3:	
Kathon CG	0,05 Gew.-%
Triethanolamin	0,45 Gew.-%

Rezept 4: Isoflavon-Creme

Phase 1:

Diglycerindiisostearat	4 Gew.-%
Vaseline 4814	10 Gew.-%
Paraffinöl P 1500	5 Gew.-%
Lunacera M	1,0 Gew.-%
Cetiol 868	5,0 Gew.-%
Methylparaben	0,15 Gew.-%
Propylparaben	0,10 Gew.-%
Sojaextrakt G	10 Gew.-%

Phase 2:

Wasser	64,3 Gew.-%
Magnesiumsulfat	0,40 Gew.-%

Phase 3:

Kathon CG	0,050 Gew.-%
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Herstellungsvorschrift für Rezept 3 und 4

Phase 1 und 2 werden separat auf 70 Grad erwärmt und danach zusammengegeben. Bis zur Abkühlung auf 35 Grad wird gerührt. Schließlich wird Phase 3 unter Rühren zugegeben.

Rezept 5: Haarwuchs-Spray

Ethanol denat.	88 Gew.-%
Calcium-Succinat	1 Gew.-%
N-Acetylcystein	0,5 Gew.-%
Glutamin	0,05 Gew.-%
Sojaextrakt G	10 Gew.-%

Rezept 6: Isoflavon-Liposome

Sojaextrakt	30%
Wasser demin	40%
Lipiddispersion durch Kavitation prozessieren.	30%

Patentansprüche

1. Mittel zur Behandlung der Haut, dadurch gekennzeichnet, daß es als Wirkstoff Isoflavon enthält.
2. Mittel nach Anspruch 1, dadurch gekennzeichnet, daß es Derivate des Isoflavons enthält.
3. Mittel nach Anspruch 2, dadurch gekennzeichnet, daß die Derivate Glykoside, Ether, Ester, Acetale des Isoflavons oder vom Isoflavon abgeleitete Säuren, Salze, Chinone, Acetale sind.
4. Mittel nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß es mindestens 0,005 Gew.-% Isoflavon oder Isoflavonderivate enthält.
5. Mittel nach Anspruch 4, dadurch gekennzeichnet, daß es 0,1 bis 0,005 Gew.-% Isoflavon oder Isoflavonderivate enthält.
6. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 4-Hydroxy-7-Glucose-Isoflavon ist.
7. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 5,7,4'-Trihydroxy-Isoflavon ist.
8. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 5,4'-Dihydroxy,

7-Glucose-Isoflavon ist.

9. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 5-Hydroxy 7,4'-Dimethoxy-Isoflavon ist.

10. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 7,4'-Dihydroxy-Isoflavon ist.

11. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 5,7'-Dihydroxy-4'-methoxy-Isoflavon ist.

12. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 3',4',5,7-Tetrahydroxy-Isoflavon ist.

13. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 3',4',5-Trihydroxy-7-methoxy-Isoflavon ist.

14. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 3',5,7-Trihydroxy-4'-methoxy-Isoflavon ist.

15. Mittel nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Wirkstoff 3',5,7-Trihydroxy-4',5',6-trimethoxy-Isoflavon ist.

16. Mittel nach einem der Ansprüche 1 bis 15, dadurch gekennzeichnet, daß es zusätzlich Sterole enthält.

17. Anwendung des Mittels nach einem der Ansprüche 1 bis 16 zur medizinischen oder kosmetischen Behandlung der Haut gegen Couperose, Besenreißern, Melanomen, Alopecie, Akne, Fetthaut und Pigmentflecken.

18. Verwendung des Mittels nach einem der Ansprüche 1 bis 17 als Haarwuchsmittel.

19. Verwendung des Mittels nach einem der Ansprüche 1 bis 17 als Radikalfänger.

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(57) Abstract <p>The invention relates to compositions and methods of managing the manifestations of the symptoms of persistent reproductive transition (or SPRT). A typical composition contemplated by the invention comprises (i) a first active ingredient comprising at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and (ii) a second active ingredient comprising (a) a mixture of remedial carbohydrates including at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least a starch, (b) choline, a source of choline, or combinations thereof, or (c) a combination of (a) and (b). If the second active ingredient of the composition comprises phosphatidyl choline, then the composition contemplated is substantially free of added beta-sitosterol.</p>			

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COMPOSITION AND TREATMENT FOR PERSISTENT REPRODUCTIVE TRANSITION SYMPTOMS

1. Field of the Invention

5 The present invention relates to compositions and methods for alleviating ailments symptomatic of premenopausal and/or menopausal disorders, associated in some way with an acute, temporary, developing, or chronic imbalance in the serum levels of endogenous, 10 gynecologically relevant substances, including certain neurotransmitters, neurotransmitter substrates and/or hormones. In particular, the present invention relates to a composition or compositions comprising a mixture of remedial carbohydrates and at least one phytoestrogen, 15 which on administration to a subject in need thereof relieves or manages the disorders, manifestations, conditions, or discomforts complained of.

2. Background of the Invention

20 Somatic, emotional, metabolic and cognitive difficulties, including sleep problems, vasomotor symptoms and mood swings, are just some of the symptoms experienced by many menopausal women and also by many premenopausal or perimenopausal women. The severity of the symptoms 25 caused by these reproductive physiological changes may differ among women of various ages (and even within women of approximately the same age). Some women have little or no discomfort, while others become socially and/or physically dysfunctional. As for women undergoing 30 menopause, fully 58 percent describe the process as "somewhat bothersome", while a third to one fifth of women found it "very bothersome" depending upon the age of the women reaching menopause. Prevention, (Aug. 1994) 78-142. The collection of such various aches, irritability, water 35 retention and other complaints experienced to some degree or another by these women whose bodies are in a state of

flux can be generally referred to as Symptoms of Persistent Reproductive Transition or SPRT.

The etiology of these reproductive physiological changes is not universally agreed upon. SPRT-related changes can affect vasomotor, cognitive, psychological and sexual functions. Specific symptoms include, but are not limited to, weight gain, sleep disorders, hot flashes, sweating, nervousness, depression, anxiety, vertigo, fatigue, arthralgia, headache, tachycardia, vaginal dryness and heavy bleeding.

It is believed that altered estrogen levels in women may play a significant role in causing SPRT-related ailments. Consequently, Hormonal Replacement Therapy (HRT) has been prescribed as a means to supplement or replace the loss of estrogen commonly observed in women experiencing SPRT-related ailments. The estrogen and endogenous hormones used in HRT, however, have undesirable side effects such as weight gain, excessive bleeding, fluid retention, and increased risk of cancer. See, Prevention, (Aug. 1994) 78-142. Safer and more effective therapies for SPRT-related ailments continue to be sought.

There are a number of articles describing the potential treatment of the somatic symptoms of menopausal women by ingestion of foods enriched with phytoestrogens or by ingestion of concentrated forms of phytoestrogens. It has been recognized that the incidence of certain somatic symptoms occurs less frequently in Chinese and Japanese women as compared to women in western countries. Substantial dietary differences exist between these Eastern and Western populations, especially related to the consumption of soy products. Consequently, it has been suggested that the estrogenic effects of phytoestrogens found in soy foods may be responsible for modifying the frequency and severity of somatic symptoms observed in

menopausal women in different countries. See, Knight, D. C. et al., *Obstetrics & Gynecology*, (1996) 87:897-904.

Phytoestrogens are naturally occurring compounds found in many foods and are defined as plant substances that are structurally or functionally similar to estradiol and consist of a number of classes, including lignans, isoflavones, coumestans and resorcylic acid lignans. Recently, a few reports have appeared purporting to observe the alleviation of some somatic symptoms of menopausal women based on the use of the isoflavonoid class of phytoestrogens. These reports, however, continue to focus solely on the use of isoflavonoids as potential agents for modifying estrogen levels to treat somatic symptoms. Most significantly, these references do not teach, disclose, or suggest any benefits to emotional, cognitive, or metabolic symptoms. Nor do these references teach, disclose, or suggest any benefits to the administration or co-administration of remedial carbohydrates to provide relief to a whole host of SPRT-related ailments.

Descriptions of methods for treating somatic symptoms of menopausal women by the administration of phytoestrogens exist. Gorbach et al., in U.S. Patent No. 5,498,631, describe a method of treating premenstrual symptoms, menopausal symptoms, or a condition related to reduced levels of endogenous estrogen in women by administering isoflavonoids, which are thought to substitute for the reduced levels of endogenous estrogen. Clearly, Gorbach et al. concentrate on the alleged estrogen-like actions of isoflavonoids. Gorbach et al. make no mention of the potential role of isoflavonoids in a composition or treatment, which also includes effective amounts of remedial carbohydrates, to alleviate a broader range of SPRT-related ailments, including those having an etiology in reduced levels of serotonin. Furthermore, Gorbach et al. do not mention the potential role of

phytoestrogens for the treatment of emotional, metabolic, or cognitive disorders.

5 In U.S. Patent No. 5,506,211 issued to Barnes et al., methods are disclosed for treating patients specifically with the isoflavone genistein to inhibit osteoclastic activity, thereby allegedly preventing osteoporosis. Similarly, Shlyankevich, in U.S. Patent No. 5,424,331, concentrates on the use of phytoestrogens as a means for regulating hormonal levels in women suffering form
10 osteoporosis. Furthermore, Hughes et al., in U.S. Patent No. 5,516,528, describe a composition comprising mammalian estrogen as a means for regulating hormonal imbalances. Neither Shlyankevich nor Hughes et al. disclose a method for alleviating common SPRT-related ailments.

15 Apart from attempting to treat solely the somatic symptoms related to reproductive physiological changes, other references have utilized chemically modified isoflavones, also referred to herein as phytoestrogen derivatives. In U.S. Patent No. 4,390,559, granted to
20 Zilliken, a composition is described using a chemically modified isoflavonoid-like compounds as antioxidants for the preservation of fats and oils. These methods and compositions do not use the naturally occurring isoflavone. Moreover, the use of excessive amounts of
25 fats and oils would inhibit the beneficial effects of remedial carbohydrates. Similarly, U.S. Patent No. 5,352,384, granted to Shen, describes a modified isoflavone that is combined with a highly insoluble fiber.

30 In U.S. Patent 4,557,927, granted to Miyake et al., a process is disclosed for enzymatically converting natural soybean glycosides to an alpha-glycosylated product in which the carbohydrate enzyme substrate is covalently bonded to the phytoestrogen. Similarly, isoflavonoid compounds can be synthesized which resemble
35 the natural isoflavones. In Patent Nos. 4,166,862, 4,163,746, 3,949,085 and 3,864,362, granted to Feuer et

al., non-natural isoflavone compounds are synthesized for use as anabolic or catabolic feed additives. Stadler nee Szoke et al., in U.S. Patent Nos. 5,043,326 and 4,826,963, describe a method for the preparation of inclusion
5 complexes of ipriflavon cyclodextrin.

Despite a great deal of effort in developing treatments for menopause, there remains an unfulfilled need for a more effective, comprehensive therapy. In particular, prior compositions for treating menopause have
10 involved the use of hormonal replacement therapy, which fall well short of addressing the needs of those women experiencing SPRT-related ailments. Quite surprisingly, it has now been found that a more general sense of well-being, as well as other benefits, is observed through a
15 regimen that affects the imbalances in the body, which imbalances are due not only to hormone or hormone-like substances but also to other substances of gynecological significance, including classes of neurotransmitters.

In mammals, the amino acid tryptophan is the precursor to serotonin synthesis in the brain. Certain
20 carbohydrates, when ingested, can increase the ratio of tryptophan to large neutral amino acids (T:LNAA) in the blood stream. An increase in the ratio of T:LNAA has been shown to result in a higher level of tryptophan in the
25 brain. A higher level of tryptophan in the brain is believed, in turn, to lead to an increase in the synthesis of endogenous serotonin. While conventional foods (e.g., a potato or a bagel) may fortuitously shift the T:LNAA ratio to a limited extent, these conventional foods also
30 contain fats, sources of protein, other fibers, or may be consumed with other foods that serve as sources of these other components. These other components of conventional foods may slow down digestion, absorption, metabolism and excretion, increase the levels of large neutral amino
35 acids, or otherwise interfere with the desired shift in the balance of specific amino acids in the blood.

It is also important to point out that while a deficiency or imbalance in serotonin levels has emerged as a leading theory behind the symptoms of premenstrual syndrome or PMS (e.g., a number of studies have shown that women with PMS may have a lower serotonin level than women without PMS), such a theory fails to account for or predict the effect of the regulation of serotonin levels (or the levels of other select neurotransmitters, such as dopamine) on premenopausal or menopausal women.

The present invention relates to the discovery that a composition comprising an effective amount of at least one phytoestrogen, in conjunction with a mixture or blend of remedial carbohydrates or in conjunction with choline or a source of choline, can alleviate, treat, or prevent SPRT-related ailments.

3. Summary of the Invention

The present invention is directed generally to compositions exhibiting surprising efficacy in alleviating conditions and/or disorders of the type that women experiencing symptoms of persistent reproductive transition (SPRT) complain of. The present invention is also directed to methods of treating, preventing, inhibiting, managing, ameliorating, or alleviating such symptoms. In particular, relief from the negative effects of the symptoms or manifestations, including somatic, emotional, metabolic, or cognitive disorders, may be achieved through dietary management using the compositions and methods of the invention.

Accordingly, the invention provides a composition for alleviating symptoms of persistent reproductive transition (SPRT) comprising (i) a first active ingredient comprising at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and (ii) a second active ingredient comprising (a) a mixture of remedial carbohydrates including at least one simple remedial

carbohydrate, at least one complex remedial carbohydrate and at least a starch, (b) choline, a source of choline, or combinations thereof, or (c) both (a) and (b), the composition being substantially free of added beta-sitosterol if the second active ingredient comprises phosphatidyl choline. In other embodiments, however, particularly those in which the second active ingredient does not comprise phosphatidyl choline, phytosterols may be added to advantage.

The invention also provides a method of alleviating the negative effects of symptoms of persistent reproductive transition (SPRT) comprising administering to a subject in need thereof an effective amount of at least a first substance comprising at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and an effective amount of at least a second substance comprising (a) a mixture of remedial carbohydrates including at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least one remedial starch, (b) choline, a source of choline, or combinations thereof, or (c) a combination of (a) and (b). Consistent with a method of dietary management, first, second, or both substances are preferably administered with a carrier, more preferably a nutritionally acceptable carrier, most preferably an edible solid, edible semi-solid, or edible liquid carrier.

The invention also contemplates a method that alleviates the negative effects of symptoms of persistent reproductive transition (SPRT) relating to emotional disorders, metabolic disorders, cognitive disorders, or combinations thereof, but not somatic disorders, comprising administering to the subject in need thereof an effective amount of at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof. In a particular embodiment, the method contemplated further comprises administering an effective

amount of a blend of two or more remedial carbohydrates, preferably a mixture of at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least one remedial starch. More preferably, the
5 method further comprises administering an effective amount of choline or a source thereof. Any route of administration can be taken. However, the oral (by mouth) or topical (transdermally, intravaginally, or the like) route is preferred. Moreover, each active component can
10 be administered sequentially, preferably within about an hour of each other, or substantially concurrently.

It has surprisingly been discovered that the compositions and methods of the invention provide great benefits to women who experience a whole range of
15 discomforts associated with SPRT. Such benefits include relief from, for example, somatic, emotional, metabolic, or cognitive associated ailments. It is believed that the compositions and methods of the invention provide relief from the symptoms by supplying particular nutrients,
20 including a combination of selected substances, having the capacity to correct (through a regimen of dietary management) adverse physiological, psychological and psychiatric states stemming from alterations primarily in endocrine synthesis and secretion accompanying a woman's
25 approach, progression and passage through a post reproductive stage during her later years of life.

To name some of the specific benefits observed from the administration of the compositions or implementation of the methods of the invention to subjects suffering from
30 SPRT-related ailments include, but are not limited to, inhibition of breakthrough bleeding, elimination of need for concurrent hormone replacement therapy, stimulation of osteoblastic activity and inhibition of the hardening of the vasculature. In other instances, it has also been
35 observed that subjects receiving treatment experience an improvement in mood, less water retention, less

irritability, or an increased ability to concentrate or remain mentally alert.

Hence, in a specific embodiment of the invention, a useful composition is disclosed for the dietary management of SPRT-related ailments, comprising at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and a mixture of blend of remedial carbohydrates. In another embodiment, the composition of the invention further comprises an amount of choline sufficient to inhibit or reverse episodes of cognitive deficit or decline, including lack of alertness or an inability to maintain premenopausal functionality, in those females unsettled by symptoms of persistent reproductive transition.

In still another embodiment, a composition is described which comprises a combination of at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and an effective amount of choline, a source of choline, or combinations thereof, independent of the presence of an effective amount of a mixture or blend of selected remedial carbohydrates.

Hence, the invention contemplates methods of using therapeutic and/or nutritional compositions for alleviating, treating, preventing, ameliorating, or managing the adverse effects of SPRT-related ailments. Broadly, such methods comprise administering a therapeutically or nutritionally effective amount of the composition to subjects in need thereof.

Other objects of the invention will become apparent to those of ordinary skill in the art upon further consideration of the entire disclosure provided herewith.

4. Glossary of Terms

The following terms appear in the present specification and are defined as follows:

Symptoms of Persistent Reproductive Transition or
SPRT - A collection of symptoms, manifestations,
disorders, complaints, discomforts, or aches and pains
which is experienced by perimenopausal or premenopausal
5 women, menopausal women, or postmenopausal women. In
particular, a subject suffering from SPRT or an SPRT-
related ailment is a woman having at least one, preferably
at least two, most preferably at least three of the
symptoms listed in Table 1, below, which symptoms are
10 categorized as somatic, emotional, metabolic, cognitive,
or nighttime symptoms.

TABLE 1. ILLUSTRATIVE
DISORDERS/SYMPTOMS/MANIFESTATIONS ASSOCIATED WITH SPRT

<u>I. Somatic</u>	
5	vaginal dryness
	hot flashes
	fluid retention
	breakthrough bleeding
	excessive bleeding
10	vertigo
	headache or migraines
	tachycardia
	libido changes (loss of sexual drive)
	sleep problems
15	joint pain
	frequent urination (incontinence)
	breast tenderness
	chills
	cold sweats (night sweats)
20	
<u>II. Emotional</u>	
	irritability
	anxiety
	lack of energy (lethargic)
25	fatigue
	mood changes (depressed mood, mood swings)
<u>III. Metabolic</u>	
	weight gain
30	marked change in appetite (increased or decreased)
	arthralgia (muscle ache)
	carbohydrate craving
	greater tendency to incur a bone fracture
35	
<u>IV. Cognitive</u>	
	forgetfulness
	recall or short term memory loss
	loss of alertness
	inability to concentrate
40	
<u>V. Nighttime or Sleep Related Symptoms</u>	
	vaginal dryness
	libido change
	sleep problems
45	chills
	incontinence (bladder control)
	night sweats

50 The sub-category of sleep problems may relate to difficulty in initially falling asleep, waking up one or more times during the night, difficulty in falling back asleep once awakening during the night, lack of quality

sleep (e.g., a fitful sleep), a feeling of grogginess in the morning, and the like.

Another subject in need of treatment, who may benefit from the invention, is a woman having episodic or chronic manifestations of at least two of the recited SPRT-related conditions (ailments, disorders and the like) and who is also experiencing changes or irregularities in her menstrual flow or cycle attributable to perimenopausal, menopausal, or postmenopausal transition. It is important to note, however, that some of the same symptoms, which are listed in Table 1, can be induced through the administration of, intake of, or exposure to pharmacological agents, chemicals, hormones and the like, or through accident, trauma, or surgery (e.g., loss or removal of some portion of the reproductive system, such as the uterus and/or one or both ovaries).

Remedial carbohydrates - Simple or complex carbohydrates, including certain forms of starch, which are rapidly digestible and which on consumption increases serotonin levels in the blood, serum, plasma, or synaptic structure(s) relative to the levels of this substance in the blood, serum, plasma, or synaptic structure(s) prior to the consumption of the remedial carbohydrate. That is, consumption of these remedial carbohydrates increases the level of endogenous serotonin synthesis. Examples of such remedial carbohydrates include, but are not limited to, dextrose, galactose, mannose, dextrin, maltodextrin, cyclodextrin, potato starch, pre-gelatinized starch, gelatinized starch, fructose, sucrose, maltose, maltotriose, maltotetraose, or mixtures thereof. It should be pointed out that the remedial carbohydrates, which are suitable for use in the invention, are substances, naturally derived or otherwise, which are deliberately added (as an admixture) to the other components of the compositions of the present invention. That is, carbohydrates, such as conventional flour, that

may be naturally present in certain foodstuffs are not considered to be remedial carbohydrates. In addition, a mixture or blend of two or more, perhaps three or more, remedial carbohydrates refers to a physical combination or admixture of two, three, or more distinct types of remedial carbohydrates. In preferred embodiments of the invention, a mixture comprising at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least one remedial starch may be utilized.

Phytoestrogens - Substances that belong to a family of compounds that are naturally found in certain plants (a natural source), including foodstuffs, especially soy and soy products. Soy proteins are a convenient source of phytoestrogens, especially soy phytoestrogens. Examples of phytoestrogen compounds include, but are not limited to, lignan, genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin, glycitein, texasin, equol, prunetin, apegenin, coumestrol, saponaretin, 7-hydroxyisoflavone, 5,7-dihydroxyisoflavone, 7,4-dihydroxyflavone, 6,7,4'-trihydroxyisoflavone, or their natural glycosylated (e.g., genistein 7-D-glucoside), acetylated, or methoxylated (e.g., genistein 4'-methyl ether) forms. Phytoestrogens can occur and be used either as the aglycon (i.e., minus the sugar moiety), ester, ether, or as the glycosylated form (i.e., glycosides). Examples, of phytoestrogen glycosides include, but are not limited to, genistin, daidzin, glycerin, saponarin and the like. Naturally obtained phytoestrogens can, of course, be prepared synthetically or semi-synthetically using conventional synthetic methods. In keeping with the desire to substantially utilize only naturally occurring active components, phytoestrogens, as used herein, do not include synthetic derivatives or analogs of the naturally obtained phytoestrogens, except as mentioned above. Such

derivatives or analogs of phytoestrogens may also be referred to herein simply as "phytoestrogen derivatives."

Choline or a Source of Choline - These terms refer to the substance choline or 2-hydroxy-N,N,N-trimethylethanaminium [$\text{HOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$], to choline salts, typically as its halide (e.g., fluoride, chloride, bromide or iodide) or hydroxide salt, or to a substance, which when broken down in the body increases circulating levels of choline. Examples of substances that provides a source of choline in vivo, include but are not limited to citicoline or CDP-choline, phosphatidyl choline and the like. Certain foods or foodstuffs may also provide a source of choline, including but not limited to, animal and plant products, such as bile, brains, lecithin and the like, egg yolks, hops, barley, belladonna, or strophanthus.

Phytosterols - Substances having a steroidal backbone or nucleus and which may be isolated from plants. An example, includes alpha-sitosterol, beta-sitosterol, gamma-sitosterol, campesterol, stigmasterol, delta-5-avenasterol, delta-7-stigmasterol, brassicasterol, lupenol, alpha-spinasterol, or the like. Phytosterols may be present naturally in certain foods and foodstuffs, which may form part of the compositions of the present invention. Phytosterols may also be deliberately added, except as noted above concerning the proviso against a combination of beta-sitosterol and phosphatidyl choline.

5. Detailed Description of the Preferred Embodiments

One of the principal ingredients of the instant compositions, phytoestrogens, is generally available as isoflavones and can be obtained from soy proteins, soybeans, vegetable protein, tempeh, tofu, miso, aburage, atuage, or koridofu. Preferred phytoestrogens include, but are not limited to, genistein, daidzein, glycerin or a combination thereof. As mentioned above, the remedial

carbohydrate mixture or blend constitutes an admixture of two or more, preferably three or more, different types of remedial carbohydrates. Preferred types of remedial carbohydrates include, but are not limited to, dextrose, dextrin, maltodextrin, mannose, pre-gelatinized starch, gelatinized starch and starch, particularly potato starch. Choline or a source thereof can be obtained from any combination of lecithin, choline chloride, choline bitartrate and choline dihydrocitrate. Choline, itself, comprises free choline, its salt, ester, acid, or synthetic or natural conjugate (e.g., CDP-choline, phosphatidyl choline and the like).

In one embodiment of the invention compositions are provided which comprise at least one phytoestrogen in an admixture with at least a second active component selected from (i) a mixture or blend of remedial carbohydrates, (ii) choline or a source thereof, or (iii) both carbohydrates and choline. A preferred composition comprises an admixture of (a) about 20-100 g of soy protein (preferably, about 20-80 g or more preferably, about 30-60 g) comprising about 20-55 mg, preferably about 30-45 mg, of soy isoflavones (i.e., one or more phytoestrogens), (b) about 20-80 g of the remedial carbohydrate mixture or blend comprising about 25-45% of dextrose, about 40-70% of maltodextrin and about 1-10% of potato starch by weight of total remedial carbohydrate, and (c) about 0-5 g of choline, preferably about 1-3 g. A more preferred composition comprises an admixture of (a) about 60 g of soy protein comprising at least about 14 to about 27 mg of genistein and at least about 12 to about 18 mg of daidzein, (b) about 50 g of a remedial carbohydrate mix comprising about 37% of dextrose, about 60% of maltodextrin and about 3% of potato starch by weight of total remedial carbohydrate, and (c) about 1 g of free choline. As discussed above, the genistein and

daidzein can be replaced by genistin and daidzin, either individually or together.

Preferably, the compositions provided are combined with a carrier, more preferably a nutritionally acceptable carrier, such as an edible solid, semi-solid, or liquid carrier, including in the form of dietary product, food, food snacks, or drink, to name a few. Preferred carriers may include sorbet, sherbet, apple sauce, or pudding. Hence, the compositions contemplated can come in many forms including, but not limited to, a dry powder, liquid concentrate, ready-to-drink, ready-to-eat, cold, ambient, hot, beverage or prepared food, e.g., a flavored drink or breakfast cereal, cereal additive (sprinkled on), pastry or baked goods (pop tart, cookie, biscuit, cracker, coffee cake, muffin), pudding or food bar, frozen product (pop tart, ice cream), cake mix, or spread.

Another embodiment of the invention is directed to a method for managing, treating, alleviating, or preventing emotional, metabolic, or cognitive disorders experienced by premenopausal, menopausal, or postmenopausal women by administering to the subject an effective amount of at least one phytoestrogen. Preferable, the subject in need of such treatment is administered about 30-60 g of soy protein, which includes about 30-45 mg of isoflavones. Most preferably, the subject is administered about 60 g of soy protein which includes about 27 mg of genistein and about 18 mg of daidzein.

When the composition or method of the invention calls for the presence of remedial carbohydrates, simple carbohydrates may be selected from dextrose, galactose, mannose, fructose, sucrose, maltose, or mixtures thereof, while complex remedial carbohydrate may be selected from dextrin, maltodextrin, cyclodextrin, maltotriose, maltotetraose, or mixtures thereof. The remedial starch may be in turn selected from potato starch, pre-

gelatinized starch, gelatinized starch, or mixtures thereof.

The subject in need of such treatment is administered the therapeutic, dietary, or nutritional composition once or twice a day, preferably in the morning and/or evening. If the composition is administered more than once a day, the administrations are preferably separated by about four to about fourteen hours, preferably, about six to about twelve hours, more preferably about eight to about ten hours. The regimen may last for only a day, a few days, or it may continue for as long as the symptoms persist (e.g., daily for 365 days of the year). More typically, however, the regimen may last anywhere from a period of about one week, two weeks, three weeks, or four weeks to a period of about one month, two months, three months, four months, five months, six months, or at least a year.

In yet another embodiment of the invention, a method is described for managing, treating, alleviating, or preventing in a subject suffering from a SPRT-related condition, which method comprises administering to the subject in need thereof an effective amount of at least one phytoestrogen and at least a second component selected from (i) a mixture or blend of remedial carbohydrates or (ii) choline or source thereof. A preferred method comprises administering to the subject an admixture of (a) about 100 g of soy protein comprising at least about 50 mg of genistin and about 30 mg of daidzin, (b) about 50 g of a remedial carbohydrate mix comprising about 35-40% of dextrose, about 50-60% of maltodextrin and about 1-5% of potato starch by weight of total remedial carbohydrate, and (c) about 1-2 g of a choline salt.

The ultimate amount of phytoestrogen, carbohydrates, and/or choline administered will ultimately vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent and its mode and route of administration; the age, health and weight of the

recipient; the nature and extent of the symptoms; the kind of concurrent treatment; and the effect desired.

As stated above, the therapeutic compositions can be prepared in the form of a ready-to-use dietary product or in the form of a dietary product concentrate. Ready-to-use products include, but are not limited to, prepared foods such as a beverage, pastry, baked goods, pudding, food bar, or frozen product. Concentrated forms include, but are not limited to a dry powder, liquid concentrate, cereal additive, cake mix, or spread.

To further illustrate the invention, specific examples are provided herewith.

6. Examples

6.1.A Typical Formulation

An edible composition is prepared from a combination of about 20-140 g (preferably about 40-100 g) of soy proteins, including about 20-90 mg (preferably about 30-55 mg) of naturally present or added isoflavones, and about 20-80 g of a mixture of remedial carbohydrates. Preferred compositions comprises about 60-120 of soy protein, including about 45-90 mg of isoflavones. The total weight of the edible composition can range from about 40 g to about 200 g. The composition contains at least two, preferably at least about three, four, or more different types of remedial carbohydrates selected from dextrose, dextrin, maltodextrin, mannose, gelatinized starch, pre-gelatinized starch, rice starch and potato starch. In addition, the edible composition may contain about 0.5-5 g of choline or a source of choline in place of the remedial carbohydrates or in addition to the remedial carbohydrates.

6.2.A Powder Suitable for Reconstitution to a Beverage

A dry powder is prepared comprising phytoestrogen, carbohydrate mix and choline, as follows: soy proteins (60 g), isoflavones (45 mg, in the form of genistein, 27 mg, and daidzein, 18 mg), carbohydrate mix (50 g, comprised of dextrose, 18.5 g, maltodextrin, 30 g, and starch, 1.5 g), choline (1 g). This powder can be mixed with about 8-24 oz. of water to provide a beverage.

6.3.A Ready-To-Drink Beverage

A flavored beverage is prepared comprising about 8-12 oz. of flavored water mixed with phytoestrogen, carbohydrate blend and choline, as follows: soy proteins (55 g), isoflavones (60 mg, in the form of genistin, 35 mg, and daidzin, 25 mg), carbohydrate blend (60 g, comprised of dextrose, 20 g, maltodextrin, 30 g, and starch, 10 g), choline (1.5 g).

6.4.A Baked Muffin

Baked muffins are prepared by adding to two cups BISQUICK, 1 cup milk, and 1 whole egg, a dry powder comprising phytoestrogen, carbohydrate mix and choline, as follows: soy proteins (60 g), isoflavones (45 mg, in the form of genistein, 27 mg, and daidzein, 18 mg), carbohydrate mixture (50 g, comprised of dextrose, 18.5 g, maltodextrin, 30 g, and starch, 1.5 g), choline (1 g). After blending, the batter is poured into muffin molds and baked in the oven at a temperature of about 300-350 degrees Fahrenheit for about 15-30 minutes.

6.5.A Powder Suitable for Reconstitution to a Beverage

A dry powder is prepared comprising phytoestrogen and choline, as follows: soy proteins (65 g), isoflavones (50 mg, in the form of genistein, 28 mg, and daidzein, 22 mg) and choline chloride (3 g). This powder can be mixed with

about 8-24 oz. of water to provide a beverage providing phytoestrogens and choline.

6.6. Rice Pudding

5 Rice pudding is prepared by adding to two cups rice pudding mix, 1 cup milk, and 1 whole egg, a dry powder comprising phytoestrogen, carbohydrate mixture and choline, as follows: soy proteins (90 g), isoflavones (70 mg, in the form of genistin, 40 mg, and glycerin, 30 mg),
10 carbohydrate mixture (50 g, comprised of mannose, 18.5 g, maltotriose, 30 g, and pre-gelatinized starch, 1.5 g), citicoline (1.5 g). After blending, the smooth batter is poured into paper cups and refrigerated for about 30 minutes to about 1 hour prior to consumption.

6.7. Treatment of Subject No. 1

15 A subject suffering from an emotional, metabolic, or cognitive disorder is given a reconstituted beverage obtained from the powder described in Example 6.5 once a
20 day in the morning or at breakfast time. The daily dosing is continued for approximately 90 days. Improvement in the alleviation of the complained of symptoms is observed after about one month from the start of the regimen. After the three month period, the subject reports complete
25 relief from fatigue, anxiety and bouts of forgetfulness.

6.8. Treatment of Subject No. 2

30 A subject suffering from any one or a combination of SPRT-related conditions is treated by the administration of an admixture of (i) phytoestrogens in the form of 60 g of soy protein containing 45 mg of soy isoflavones (in turn containing 27 mg genistein and 18 mg daidzein); (ii)
35 20-80 g of a mixture of remedial carbohydrate including approximately 25 to 45% of dextrose, 40 to 70% of maltodextrin and 1 to 10% of potato starch; and (iii) approximately 0.5 To 5.0 g of free choline (base) or the

calculated equivalent as a choline salt, ester, acid or synthetic or natural conjugate. The admixture is administered once or twice daily, preferably administered between a 6 to 12 hour period for 365 days per year or as long as symptoms of SPRT persist.

6.9. Treatment of Subject No. 3

A subject suffering from an SPRT-related conditions is given approximately 50 g of remedial carbohydrate blend containing approximately 37% of dextrose, 60% of maltodextrin and 3% of potato starch administered in conjunction with a choline source and phytoestrogen source. The compositions is administered twice a day preferably once in the morning and once in the evening for about six months or as long as the SPRT-related symptoms persist.

6.10. Treatment of Subject No. 4

A subject suffering from vertigo, weight gain and lapses in memory is treated by receiving approximately 1.0 g of free choline administered in conjunction with a remedial carbohydrate blend and phytoestrogen source according to the muffin of Example 6.4. The muffin is administered once a day preferably in the morning at breakfast for 255 days. The subject is symptom free after the treatment period.

Hence, the foregoing examples illustrate the successful management, through the administration of a diet of nutritional supplements, of a wide range of symptoms associated with perimenopausal, menopausal, or postmenopausal ailments experienced by women in their middle to late years of life.

It should be apparent to those of ordinary skill in the art that other embodiments of the invention may be readily contemplated in view of the teachings of the present specification. Such embodiments, although not

specifically disclosed, nevertheless fall within the scope
and spirit of the invention. Hence, the invention should
not be construed as being limited to the specific
embodiments provided, which invention is limited solely
5 by the claims that follow.

WHAT IS CLAIMED IS:

1.A composition for alleviating symptoms of persistent reproductive transition (SPRT) comprising (i) a first active ingredient comprising at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and (ii) a second active ingredient comprising (a) a mixture of remedial carbohydrates including at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least a starch, (b) choline, a source of choline, or combinations thereof, or (c) a combination of (a) and (b), said composition being substantially free of added beta-sitosterol if the second active ingredient comprises phosphatidyl choline.

15

2.The composition of claim 1 which further comprises a nutritionally acceptable carrier.

3.The composition of claim 2 in which said carrier comprises an edible solid carrier.

20

4.The composition of claim 2 in which said carrier comprises an edible liquid carrier.

5.The composition of claim 1 in which said phytoestrogen comprises a soy phytoestrogen.

25

6.The composition of claim 1 in which said phytoestrogen is obtained from its natural source or from a synthetic or semi-synthetic process.

30

7.The composition of claim 5 in which said phytoestrogen is obtained from soybean or a soy product selected from tofu, miso, aburage, atuage, or koridofu.

35

8. The composition of claim 1 in which said phytoestrogen comprises a lignan, genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin, glycitein, texasin, equol, prunetin, apegenin, coumestrol, saponaretin, 7-hydroxyisoflavone, 5,7-dihydroxyisoflavone, 7,4-dihydroxyflavone, 6,7,4'-trihydroxyisoflavone, or their natural glycosylated, acetylated, or methoxylated forms.

9. The composition of claim 8 in which said phytoestrogen comprises genistin, daidzin, glycerin, or saponarin.

10. The composition of claim 1 in which said remedial carbohydrates on consumption increases the level of endogenous serotonin synthesis.

11. The composition of claim 1 in which said simple remedial carbohydrate is selected from dextrose, galactose, mannose, fructose, sucrose, maltose, or mixtures thereof.

12. The composition of claim 1 in which said complex remedial carbohydrate is selected from dextrin, maltodextrin, cyclodextrin, maltotriose, maltotetraose, or mixtures thereof.

13. The composition of claim 1 in which said remedial starch is selected from potato starch, pre-gelatinized starch, gelatinized starch, or mixtures thereof.

14. The composition of claim 1 in which the second active ingredient comprises a mixture of remedial carbohydrates including at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least a remedial starch.

15.The composition of claim 1 in which the second active ingredient comprises choline, a source of choline, or combinations thereof.

5 16.The composition of claim 1 in which the second active ingredient comprises a blend of three or more remedial carbohydrates and further comprises choline, a source of choline, or a combination thereof.

10 17.The composition of claim 14 which contains about 20 to about 80 g of said remedial carbohydrates.

15 18.The composition of claim 14 in which said mixture of remedial carbohydrates comprises about 25 to about 45% dextrose, about 40 to about 70% maltodextrin and about 1 to about 10% potato starch.

20 19.The composition of claim 17 which contains about 50 g of said remedial carbohydrates comprising about 37% dextrose, about 60% maltodextrin and about 3% potato starch.

25 20.The composition of claim 1 which contains about 20 to about 100 g of soy protein.

 21.The composition of claim 1 which contains about 20 to about 55 mg phytoestrogen.

30 22.The composition of claim 21 in which said phytoestrogen comprises about 14 to about 27 mg of genistein and about 12 to about 18 mg of daidzein.

35 23.A method of alleviating the negative effects of symptoms of persistent reproductive transition (SPRT) comprising administering to a subject in need thereof an effective amount of at least a first substance comprising

at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof and an effective amount of at least a second substance comprising (a) a mixture of remedial carbohydrates including at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least one remedial starch, (b) choline, a source of choline, or combinations thereof, or (c) both (a) and (b).

24. The method of claim 23 in which the first, second, or both substances are administered with an edible carrier.

25. The method of claim 23 which is administered daily for a period of about one week, two weeks, three weeks, or four weeks.

26. The method of claim 23 which is administered daily for a period of about one month, two months, three months, four months, five months, six months, or at least a year.

27. The method of claim 23 which is administered once daily.

28. The method of claim 23 which is administered twice daily.

29. A method of alleviating the negative effects of symptoms of persistent reproductive transition (SPRT) relating to metabolic disorders, cognitive disorders, or combinations thereof, but not emotional disorders, somatic disorders, or osteoporosis, comprising administering to the subject in need thereof an effective amount of at least one phytoestrogen, a source of at least one phytoestrogen, or combinations thereof.

30.The method of claim 29 which further comprises administering an effective amount of a blend of two or more remedial carbohydrates.

5 31.The method of claim 29 which further comprises administering an effective amount of a mixture comprising at least one simple remedial carbohydrate, at least one complex remedial carbohydrate and at least one remedial starch.

10 32.The method of claim 29 which further comprises administering an effective amount of choline, a source of choline, or combinations thereof.

15 33.The method of claim 29 in which the administration is performed orally.

 34.The method of claim 29 in which the administration is performed topically.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20957

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 33/12, 65/00, 43/16, 47/40

US CL : 514/60, 456, 642, 514

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/60, 456, 642, 514

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

A Modern Herbal

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,424,331 A (SHLYANKEVICH) 13 JUNE 1995. See whole document.	1-34
Y	US 5,498,631 A (GORBACH et al.) 12 MARCH 1996. See whole document.	1-34
Y	US 3,949,085 A (FEUER et al.) 06 APRIL 1976. See whole document.	1-34
Y	US 4,166,862 A (FEUER et al.) 04 SEPTEMBER 1979. See whole document.	1-34
Y	US 5,516,528 A (HUGHES et al.) 14 MAY 1996. See whole document.	1-34

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No.
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B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, AGRICOLA, WPIDS

Search terms: phytoestrogen, estrogen, menopause, PMS, premenstrual, melatonin, genistein, genistin, saponarin, glycerin

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US96/12563</p> <p>(22) International Filing Date: 7 August 1996 (07.08.96)</p> <p>(30) Priority Data: 08/512,192 7 August 1995 (07.08.95) US</p> <p>(71) Applicant: FERMALOGIC, INC. [US/US]; 2201 W. Campbell Park Drive, Chicago, IL 60612 (US).</p> <p>(72) Inventors: WEBER, J., Mark; 3100 N. Lake Shore Drive #2103, Chicago, IL 60657 (US). CONSTANTINO, Andreas; 1516 E. Bailey, Naperville, IL 60565 (US). HESSLER, Paul, E.; 6815 W. 32nd Street, Berwyn, IL 60402 (US).</p> <p>(74) Agents: NORTHRUP, Thomas, E. et al.; Dressler, Goldsmith, Milnamow & Katz, Ltd., Suite 4700, 180 N. Stetson, Chicago, IL 60601 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>
(54) Title: A PROCESS OF PREPARING GENISTEIN		
(57) Abstract		
<p>The present invention provides a process of preparing the isoflavone genistein. Genistein is prepared by fermenting the bacteria <i>Saccharopolyspora erythraea</i> in the presence of a soy-based substrate. A process of recovering genistein from a bacterial fermentation medium using alkaline, organic solvent extraction is also provided.</p>		

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A PROCESS OF PREPARING GENISTEIN

Technical Field of the Invention

5 The field of this invention is genistein production. More particularly, the field of this invention is genistein production using fermentation of the bacteria *Saccharopolyspora erythraea* on a soy-based substrate.

Background Of The Invention

10 Genistein is an isoflavone. Isoflavones are a subclass of flavonoids, natural products typically isolated in glycosylated form from plants. The aglycone is the biologically active form that has the most medical and commercial interest.

15 The glycosylated form of genistein is known as genistin. The preparation of genistein must include a step in which the core isoflavone structure (genistein) is separated from the glucose moiety. Genistin, which is found in soybeans, is converted to the biologically active form, genistein, through the action of a beta-glucosidase enzyme.

20 Demand for genistein is expected to increase significantly in the near future. Genistein is becoming an established research tool as an inhibitor of protein tyrosine kinase, an enzyme involved in increased cell proliferation. Protein tyrosine kinase (PTK), is one of genistein's well studied biological targets. PTK is known to give cells a proliferative advantage. A variety of
25 oncogenes of the Src family have been shown to have tyrosine kinase activity. Genistein is a PTK inhibitor. Genistein inhibited the PTK activities of EGF-receptor and pp60v-src with an ID50 of 6 and 7 mg/ml respectively in experiments using purified components, but in intact A431 cells 40 mg/ml were required to inhibit the cellular phosphotyrosine levels. Genistein is reported to be
30 an inhibitor of eukaryotic DNA topoisomerase (topo) I and II.

35 Genistein is used as a chemopreventive agent in animal studies, is required for clinical trials as a dietary supplement; and likely has application as a chemotherapeutic agent when coupled to anti-tumor specific antibodies.

Populations consuming soybeans show reduced incidence of breast, colon, and prostate cancer. Initial animal studies suggested that two isoflavones, genistein and daidzein, may be the active ingredients of soybeans that function as chemopreventive agents. Genistein injected into neonatal rats reduces
5 dimethylbenz(a)anthracene (DMBA)-induced mammary tumor induction by about 50%. Either genistein or daidzein provides some protection against N-methyl N-nitrosurea (MNU)-induced mammary tumors in rats. Although genistein was effective in reducing both the tumor incidence and multiplicity, daidzein reduced only multiplicity in the later studies.

10
Currently genistein is being tested by the National Cancer Institute against colon carcinogenesis, as part of the Chemoprevention Screening Program, and early results show effectiveness against colon papillomas. Clinical trials to identify the metabolism and pharmacokinetics of genistein (phase I) are also being
15 currently performed.

A recent study identified genistein as a potent antitumor agent (when coupled with antitumor specific antibodies) against colon carcinogenesis of human B-cell leukemia in immunodeficient mice. The B43-genistein
20 immunoconjugate at less than one-tenth the maximum tolerated dose killed more than 99% of human leukemia cells. The postulated mechanism of genistein's action in this study was that of PTK inhibitor.

25 Because of the potential clinical use of genistein as a chemopreventive and/or chemotherapeutic agent it is becoming essential to produce this isoflavone and some of its promising structural analogs in larger quantities and to reduce its price. Genistein is currently expensive to produce using current technology. Chemically synthesized genistein or genistein extracted from soybeans currently can cost up to \$5,000 a gram. However, the compound is not produced routinely
30 in kilogram quantities at present.

The present invention provides an inexpensive process for the large scale production of genistein wherein *Saccharopolyspora erythraea*, the organism that is used in the commercial production of erythromycin, is fermented on a soy-
35 based fermentation medium.

Brief Summary of The Invention

The present invention provides a process of preparing genistein comprising fermenting the bacteria *Saccharopolyspora erythraea* on a soy-based substrate. The genistein that is formed by this fermentation is then recovered from the fermentation medium. In accordance with this process, *Saccharopolyspora erythraea* is cultured for a period of time and under culture conditions sufficient for genistein formation. Suitable culture conditions include a temperature of from about 10°C to about 50°C in an aqueous, oxygenated medium having a pH value of from about 4.0 to about 9.0, which medium contains organic nutrients, vitamins and inorganic salts sufficient to maintain *Saccharopolyspora erythraea* viability. More preferably, the temperature is from about 20°C to about 30°C and the medium has a pH value of from about 6.0 to about 8.0. A preferred time of fermentation is from about 20 hours to about 250 hours.

Genistein is recovered from the fermentation or culture medium by extraction in a water-immiscible organic solvent at a pH value of from about 8.0 to about 11.0. Preferably, the pH value is from about 9.0 to about 10.0. A preferred organic solvent is ethyl acetate.

In another aspect, the present invention provides a process of recovering genistein from a bacterial fermentation medium comprising extracting genistein from the medium in a water-immiscible organic solvent at a pH value of from about 8.0 to about 11.0 and, preferably from about 9.0 to about 10.0. A preferred organic solvent is the same as set forth above. In a preferred embodiment, the bacterial fermentation is a fermentation of *Saccharopolyspora erythraea*.

Detailed Description of the Invention

I. The Invention

The present invention provides an inexpensive, large-scale process for preparing the isoflavone genistein. That process is based on the surprising discovery that genistein is produced during fermentation of the bacteria *Saccharopolyspora erythraea*, which is used in the commercial production of the antibiotic erythromycin. The present inventors have further discovered that

genistein is recovered from that fermentation along with erythromycin during alkaline, organic solvent extraction.

5 Typically, industrial fermentations are designed for the production of a single compound of interest such as an antibiotic. This compound is traditionally a biosynthetic product of the microorganism, referred to as a secondary metabolite. The growth medium is thought of strictly as a source of carbon and nitrogen and other necessary nutrients for the growth of the organism and production of the natural product from primary metabolic precursors produced by the microorganism.

10 The present disclosure that genistein is produced in the erythromycin fermentation enables one to recognize that the growth medium itself can be a significant source of additional products which can be used to add value to the overall fermentation.

20 The process of the present invention has advantages over other means for preparing genistein. When compared to chemical synthetic procedures, the present process is more efficient, less costly and capable of producing genistein in large-scale quantities. When compared to procedures involving the extraction of genistein from soy beans *per se*, the present process has the advantage of selectively producing genistein as opposed to its relatively inactive glycosylated precursor, genistin. This is true because *Saccharopolyspora erythraea* produces and secretes, during fermentation a unique form of a beta-glucosidase, which enzyme catalyses the removal of the glucose from genistin to form genistein.

25 This is highly advantageous not only because it is genistein, and not genistin, that is the biologically active compound but also because genistein (not genistin) is extractable from the fermentation medium broth under the same conditions (e.g., alkaline pH and organic solvent) that are used to recover erythromycin. It is surprising and unexpected that the same solvent and pH can be used to extract both genistein and erythromycin.

30 II. Process of Preparing Genistein

35 The present invention therefore provides a process of preparing genistein comprising fermenting *Saccharopolyspora erythraea* on a soy-based substrate.

As used herein, the term "fermenting" or its grammatical equivalents, refers to the process of culturing *Saccharopolyspora erythraea* in a medium that provides nutrients, vitamins and inorganic salts sufficient to maintain viability of the bacteria such that it will assimilate medium constituents (e.g., carbon and nitrogen) and produce erythromycin. Suitable media and conditions for bacterial fermentation are well known in the art. By way of example, United States Patent No. 2,653,899, the disclosure of which is incorporated herein by reference, discloses the fermentation of *Streptomyces erythreus* so as to produce erythromycin.

The fermentation of *Saccharopolyspora erythraea* to produce genistein in accordance with the present invention comprises culturing *Saccharopolyspora erythraea* for a period of time and under culture conditions sufficient for genistein formation. Culture conditions include, as is well known in the art, temperature and composition of the culture medium such as pH, nutrient levels, osmolality and the like.

As is also well known in the art, a preferred medium is an aqueous medium that is oxygenated. That medium contains assimilatable sources of carbon and nitrogen. Exemplary sources of carbon include, but are not limited to, carbohydrates such as starch, sugars (e.g., arabinose, fructose, galactose, maltose, sucrose) and organic salts (e.g., sodium acetate, sodium citrate, sodium malate). One of skill in the art can readily determine the optimum concentrations of such carbon sources in the culture medium.

The nitrogen source of the culture medium must include a soy-based substrate. As used herein, the term "soy-based substrate" refers to a substrate of any form that includes or is derived from soybeans. That soy-based substrate can be soybean meal, soybean flour, soybean oil, soybean grits, and the like. Such soy-based substrates are commercially available. Exemplary substrates and their commercial source are set forth hereinafter in the Examples.

The culture or fermentation medium can contain assimilatable nitrogen sources in addition to the soy-based substrate. Exemplary such additional nitrogen sources include, but are not limited to, corn steep, amino acid mixtures, casein, peptones and the like.

5 The culture medium further comprises inorganic salts that serve to provide any necessary cofactors for cell viability, genistein production and erythromycin production as well as for maintaining osmolarity within normal limits. Typically, as is well known in the art, those salts include sodium, potassium, chloride, magnesium calcium and the like. One of ordinary skill can readily determine the optimum levels of such inorganic salts. The pH of the culture medium is maintained at a suitable level for fermentation. A preferred medium pH value is from about 4.0 to about 9.0 and, more preferably from about 6.0 to about 8.0. Medium pH can be maintained with the use of buffers as needed.

15 As is well known in the art, fermentation can be performed over a wide range of temperatures. In a preferred embodiment, fermentation temperature is from about 10°C to about 45°C and, more preferably from about 20°C to about 40°C.

20 Fermentation is maintained for a period of time sufficient for genistein formation. That time will vary, as is well known in the art, on culture conditions, concentration of the bacteria and the like. Under culture conditions such as set forth above, suitable times typically range from about 20 hours to about 250 hours.

25 Genistein, formed from the fermentation of *Saccharopolyspora erythraea* in accordance with a process of the present invention is recovered from the fermentation or culture medium. Any means of recovering genistein can be used. It is preferred, however, to recover genistein using conditions similar to those used to recover erythromycin from the same medium. In accordance with this preferred embodiment and, as set forth below, genistein is recovered from the medium using organic solvent extraction at an alkaline pH.

30 III. Process of Recovering Genistein

In another aspect, therefore, the present invention provides a process of recovering genistein from a bacterial fermentation medium that contains genistein, the process comprising extracting the genistein from the fermentation medium in an organic solvent at a pH value of from about 8.0 to about 11.0.

5 In accordance with the process, the fermentation medium is adjusted to an alkaline pH by the addition of an alkalinizing salt. The alkaline pH can range from a pH value of about 8.0 to a pH value of about 11.0. More preferably, the pH value is from about a pH value of 9.0 to a pH value of about 10.0 and, even more preferably a pH value of about 9.5.

10 Any alkalinizing salt can be used so long as that salt does not interfere with the isolation and biological stability of genistein. An exemplary and preferred alkalinizing salt is sodium hydroxide.

15 The alkalized fermentation medium is then extracted with an organic solvent. That organic solvent is preferably water immiscible. Any organic solvent can be used so long as that solvent does not interfere with the isolation or biological stability of genistein. Means for determining suitable such solvents are well known in the art and within the knowledge of one of ordinary skill in the art. Exemplary and preferred organic solvent include, but are not limited to alkyl esters of fatty acids (e.g., ethyl acetate, amyl acetate) chlorinated hydrocarbons (e.g., chloroform, ethylene dichloride), ethers, ketones and alcohols (e.g., butanol, amyl alcohol). Particularly preferred organic solvents are alcohols such as n-butanol and alkyl fatty acid esters such as amyl acetate and ethyl acetate.

20 Genistein is isolated and purified from the organic solvent extract (solvent fraction) using standard procedures well known in the art. By way of example, the solvent fraction is extracted with acidified water to remove the erythromycin: the genistein stays with the organic fraction and the erythromycin is extracted into the water fraction. Genistein is then isolated from the solvent using, typically, solvent evaporation and high pressure liquid chromatography (HPLC). A detailed description of the recovery of genistein using a process of the present invention is set forth hereinafter in the Example.

30 The following Example illustrates preferred embodiments of the present invention and is not limiting of the specification and claims in any way.

35 **Example 1: Microbial Production Of Genistein**

A variety of bacterial strains were studied for their ability to produce genistein. Three of those strains (Actinomycete bacteria) were reported to

produce isoflavones and were purchased from the American Type Culture Collection (ATCC). Those three strains were *Streptomyces roseolus* (ATCC 31047), and *Micromonospora halophytica* (subspecies *halophytica* and *nigra*) (ATCC 27596 and ATCC 33088). Control strains (not reported to produce isoflavones) studied were *Saccharopolyspora erythraea* (ATCC 11635), an erythromycin producer, *Streptomyces lividans* TK21 (Hopwood et al., 1985), an actinorhodin producer, *Streptomyces hygroscopicus* (ATCC 29253) an ascomycin producer, *Streptomyces glaucescens* NRLL, a tetracenomycin producer, and *E. coli* DH5alpha.

10

Various strains were grown (fermented) in eight different media as set forth below.

1. AVMM (disclosed in U.S. Patent No. 5,141,926, the disclosure of which is incorporated herein by reference) - asparagine (5 g), glucose (20 g), phosphate buffer at pH 7.0, essential vitamins (trace) and water (1 liter).
2. STB - Soytone Broth (Difco), yeast extract, NaCl (2.5 g), CaCO₃ (3.5 g), soluble starch (20 g) trace elements solution (0.25 ml) and water (1 liter).
3. SFB - SoyFluff Broth (Central Soya), yeast extract, NaCl (2.5 g), CaCO₃ (3.5 g), soluble starch (20 g) trace elements solution (0.25 ml) and water (1 liter).
4. SGB - NutraSoyaGrits (Cargill), yeast extract, NaCl (2.5 g), CaCO₃ (3.5 g), soluble starch (20 g) trace elements solution (0.25 ml) and water (1 liter).
5. G1 - NZ amine (20 g), yeast extract (10 g), soluble starch (20 g), NaCl (2.5 g), CaCO₃ (3.5 g), trace elements solution (1.0 ml), 50% glucose solution (20 ml) and water (980 ml).
6. SGGP - Bactopeptone (4 g), yeast extract (4 g), glycine (2 g), MgSO₄ (0.5 g), 0.5 M KH₂PO₄ (20ml), 50% glucose solution (20 ml) and water (960 ml).
7. SOD - tryptone (20 g), yeast extract (5 g), NaCl (0.5 g), 50% glucose solution (20 ml) and water (1 liter).
8. GM - beef extract (0.3%), tryptone (0.5%), dextrose (0.1%), soluble starch (2.4%) and water (1 liter).

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Various bacterial strains were fermented in 50 ml of the various media at 30°C for 96 hours. 20 mls of each fermentation media were then extracted with

an equal volume of ethyl acetate. The solvent extract was evaporated to dryness, resuspended in about 100 microliters of ethyl acetate and subjected to HPLC. Extracts were examined for the presence of genistein and other isoflavones. The identification of genistein in the fermentation medium was made using standard
5 gas chromatography/mass spectroscopy (GC/MS) procedures well known in the art.

None of the bacterial strains produced isoflavones when fermented in media that did not contain a soy-based substrate (i.e., AVMM, G1, SGGP, SOD,
10 GM). These results were particularly surprising and unexpected with regard to those strains previously reported to produce isoflavones such as *Streptomyces roseolus* (ATCC 31047), and *Micromonospora halophytica* (subspecies *halophytica* and *nigra*) (ATCC 27596 and ATCC 33088). The present results bring into question the claims of U.S. Patent No. 3,973,608 to Umezawa et al.,
15 which discloses that *Streptomyces roseolus* produces isoflavone during fermentation. Although that patent disclosed soy-based fermentation media as a suitable medium, other non-soy-based media were disclosed as being suitable for isoflavone production.

In contrast, genistein was produced when *Streptomyces roseolus*, and
20 *Micromonospora halophytica* were fermented in media (STB, SFB and SGB) containing a soy-based substrate. Surprisingly, however, the fermentation of *Saccharopolyspora erythraea*, a strain not previously reported to produce isoflavones, also produced genistein in significant amounts when fermented in
25 the presence of a soy-base substrate.

Extensive chemical analyses were performed on extracts from the *Streptomyces roseolus* and *Saccharopolyspora erythraea* fermentations. Briefly,
30 purified material collected from HPLC was subjected to GC/MS analysis. The results showed that the material corresponding to the peak eluting at 15 min. was genistein. These findings were based on a comparison between the mass spectrum of material isolated from the medium and an authentic genistein standard.

35 Identity of the material was also checked against apigenin. Apigenin is the flavanone equivalent of genistein, having the same molecular weight and

substitution pattern. Its mass spectrum is identical to that of genistein, except that the two compounds were distinguishable by the significantly longer elution time of apigenin (23:12) as opposed to genistein's elution time (20:53). The two compounds could also be easily separated by HPLC.

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Genistein was not present in the uninoculated broth containing soy-based substrates and was not produced by fermentation of any bacteria in media without a soy-based substrate. These findings, taken together, show that genistein is not produced *de novo* by the bacteria. This finding is in direct contradistinction to previous teachings about the bacterial production of genistein (See, e.g., U.S. Patent No. 3,973,608).

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Although not wishing to be bound by a particular theory, it is believed that genistein is produced during fermentation through the beta-glucosidase catalyzed removal of glucose from genistin present in the soy-based substrate. The beta-glucosidase is likely produced by *Saccharopolyspora erythraea* and secreted into the fermentation medium where it acts on genistin. Beta-glucosidases are a family of substrate-specific enzymes that serve to catalyze the removal of sugar moieties from particular substrates (See, e.g., MacDonald et al., *Applied and Environmental Microbiology*, 47:350, 1984). Although beta-glucosidases have been reported to occur in over 400 microorganisms, there are no reports of any beta-glucosidase activity in *Saccharopolyspora erythraea* (See, e.g., Schliemann, *Pharmazie*, 38:1083, 1983). An examination of the report of Schliemann also reveals that there is no pattern to the particular type (i.e., substrate specificity) of beta-glucosidase amongst strains of bacteria. Thus, the observation that the fermentation of *Streptomyces griseus* on soybean meal resulted in genistein formation is, in no way, predictive of the present invention.

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Saccharopolyspora erythraea produced 2.5 mg of genistein/ml of medium. The amount of glycosylated isoflavones (e.g., genistin) in North American soybeans is reported to be in the range of about 2.5 to 5 mg of glycosylated isoflavone/g dry soybean weight. Thus, a process of the present invention results in a production and recovery of genistein of about 25% of the genistein available. A typical industrial producer of erythromycin would use about 2,000 kg of crushed soybeans per tank (100,000 L) per week. This would potentially lead to the production of 5 kg of genistein per tank per week. A

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medium scale producer of erythromycin could have 10 dedicated tanks for erythromycin production, and would therefore be capable of producing 50 kg of genistein per week.

5 Genistein was efficiently extracted from *Saccharopolyspora erythraea* fermentations at a pH of 9.5. This is significant because erythromycin is also extracted from *Saccharopolyspora erythraea* fermentations at this elevated pH (See, U.S. Patent 2,653,899, the disclosure of which is incorporated herein by reference). This means that in the industrial process genistein is co-purified with
10 erythromycin, at least through the first step. Genistein remains in the ethyl acetate fraction after the erythromycin is further purified by back extraction into acidified water. Genistein in the solvent fraction is effectively highly purified in the solvent distillate. Thus, not only are large quantities of genistein produced, but it is in a highly purified and concentrated state.

15 The present disclosure that genistein can be extracted from a bacterial fermentation medium at an alkaline pH in an organic solvent is surprising in view of the structure of genistein as well as reported methods of genistein isolation and recovery. As is well known, genistein, chemical name 4',6,7-
20 trihydroxyisoflavone, contains three hydroxyl groups. As is also well known, those hydroxyl groups tend to lose their hydrogen atoms and become negatively charged at alkaline pH values. As the pH increases, the solubility of genistein in an organic (non-polar) solvent would thus decrease and genistein should not be recoverable in that organic solvent. Indeed, all previous reports of genistein
25 isolation in organic solvents utilized an acidified environment (See, e.g., Chimura et al., *The Journal of Antibiotics*, 28:619, 1975; U.S. Patent Nos. 3,914,184 and 3,973,608; and Ogawara et al., *The Journal of Antibiotics*, 39:606, 1986).

WHAT IS CLAIMED IS:

1. A process of preparing genistein comprising fermenting
Saccharopolyspora erythraea on a soy-based substrate.
2. The process of claim 1 further comprising recovering the
genistein.
3. The process of claim 1 wherein *Saccharopolyspora erythraea* is
cultured for a period of time and under culture conditions sufficient for genistein
formation.
4. The process of claim 3 wherein *Saccharopolyspora erythraea* is
cultured at a temperature of from about 10°C to about 45°C in an aqueous,
oxygenated medium having a pH value of from about 4.0 to about 9.0, which
medium contains organic nutrients, vitamins and inorganic salts sufficient to
maintain *Saccharopolyspora erythraea* viability.
5. The process of claim 4 wherein the temperature is from about
20°C to about 30°C and the medium has a pH value of from about 6.0 to about
8.0.
6. The process of claim 3 wherein the period of time is from about
20 hours to about 250 hours.
7. The process of claim 2 wherein genistein is recovered by
extracting genistein from a fermentation medium in a water-immiscible organic
solvent at a pH value of from about 8.0 to about 11.0.
8. The process of claim 7 wherein the pH value is from about 9.0 to
about 10.0.
9. The process of claim 7 wherein the organic solvent is ethyl
acetate.

10. A process of recovering genistein from a bacterial fermentation medium comprising extracting genistein from the medium in an organic solvent at a pH value of from about 8.0 to about 11.0.
- 5 11. The process of claim 10 wherein the pH value is from about 9.0 to about 10.0.
12. The process of claim 10 wherein the organic solvent is ethyl acetate.
- 10 13. The process of claim 10 wherein the bacterial fermentation is a *Saccharopolyspora erythraea* fermentation.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12563

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 17/06

US CL : 435/126, 252.1, 822

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/126, 252.1, 822

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,124,258 A (ARISON ET AL.) 23 June 1992, see Example 1.	1, 3-7
X	US 5,140,042 A (ARISON ET AL.) 18 August 1992, see Examples.	1, 3-7
X	US 5,141,926 A (WEBER ET AL.) 25 August 1992, see Examples.	1, 3-7
X	US 5,192,671 A (ARISON ET AL.) 09 March 1993, see Examples.	1, 3-7
A	US 4,366,248 A (ZILLIKEN) 28 December 1982, see entire document.	1-13

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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(21) International Application Number: PCT/US94/04189 (22) International Filing Date: 15 April 1994 (15.04.94) (30) Priority Data: 08/049,006 16 April 1993 (16.04.93) US (71) Applicant: TUFTS UNIVERSITY SCHOOL OF MEDICINE [US/US]; 136 Harrison Avenue, Boston, MA 02111 (US). (72) Inventors: GORBACH, Sherwood, L.; 429 Beacon Street, Chestnut Hill, MA 02115 (US). GOLDIN, Barry, R.; 38 Adella Avenue, West Newton, MA 02165 (US). ADLER-CREUTZ, Herman; Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, FIN-00290 Helsinki (FI). (74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS (57) Abstract A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.		

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- 1 -

METHOD FOR TREATMENT OF MENOPAUSAL
AND PREMENSTRUAL SYMPTOMS

Background of the Invention

5 The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

 It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs
10 prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types
15 of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occurring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of
20 hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

 The inventors have found that isoflavonoids, which
25 are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that
30 the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects. Isoflavonoids which may be administered according to the
35 invention include genistein, daidzein, Biochanin A,

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formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

- 3 -

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

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Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

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Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440 (n=3)	. .	32.1 (n=12)
Daidzein	2600 (n=10)	216 (n=21)	40.5 (n=12)
Equol	2600 (n=10)	62.8 (n=21)	44.2 (n=12)
Oestrone (postmenstru al)	4.48 (n=9)	. .	4.48 (n=10)
Oestradiol (postmenstru al)	0.76 (n=9)	. .	0.94 (n=10)
Oestriol (postmenstru al)	4.48 (n=9)	. .	4.44 (n=10)

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CLAIMS

1. Use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen.
5
2. The use of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.
- 10 3. The use of claim 1 wherein said isoflavonoid is in a unit dosage of at least 30 mg.
4. The use of claim 1 wherein genistein and daidzein isoflavonoids are present in said medicament.
- 15 5. The use of claim 4 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.
6. The use of claim 1 wherein said medicament is in the form of a dietary product.
- 20 7. The use of claim 6 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.
8. The use of claim 6 wherein said dietary product is a confectionery bar containing said isoflavonoid.
- 25 9. The use of claim 6 wherein said dietary product is a cereal containing said isoflavonoid.

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10. The method of claim 6 wherein said dietary product is a biscuit containing said isoflavonoid.

11. The method of claim 6 wherein said dietary product is a beverage containing said isoflavonoid.

5 12. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

10 13. The dietary product of claim 12 comprising genistein and daidzein isoflavonoids.

14. The dietary product of claim 12 wherein the food carrier is a confectionery bar.

15 15. The dietary product of claim 12 wherein the food carrier is a cereal.

16. The dietary product of claim 12 wherein the food carrier is a biscuit.

17. The dietary product of claim 12 wherein the food carrier is a beverage.

20 18. The dietary product of claim 12 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

19. The dietary product of claim 18 comprising at least about 30 mg isoflavonoids per serving.

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20. The dietary product of claim 13 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

INTERNATIONAL SEARCH REPORT

International application No.
PC1/US94/04189

A. CLASSIFICATION OF SUBJECT MATTER IPC(5) : A61K 31/35- US CL : 514/456, 899 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 514/456, 899 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS AND CAS ONLINE: ISOFLAVIN7, PMS, ESTRO7, PREMENSTRUAL				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X --- Y	US, A, 3,864,362 (FEUER ET AL.) 04 FEBRUARY 1975, COLUMN 1, LINE 33 - COLUMN 2, LINE 44.	1-20 ----- 1-20		
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.				
<table border="0"> <tr> <td> * Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed </td> <td> *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *Z* document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *Z* document member of the same patent family
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *Z* document member of the same patent family			
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(21) International Application Number: PCT/AU00/00384 (22) International Filing Date: 27 April 2000 (27.04.00) (30) Priority Data: PQ 0083 28 April 1999 (28.04.99) AU (71) Applicant (for all designated States except US): NOVOGEN RESEARCH PTY LTD [AU/AU]; 140 Wicks Road, North Ryde, NSW 2113 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): KELLY, Graham, Edmund [AU/AU]; 1,47 Coolawin Road, Northbridge, NSW 2063 (AU). HUSBAND, Alan, James [AU/AU]; 13 Marana Road, Northbridge, NSW 2063 (AU). (74) Agents: STEARNE, Peter et al.; Davies Collison Cave, Level 10, 10 Barrack Street, Sydney, NSW 2000 (AU).		(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: CARDIOVASCULAR AND BONE TREATMENT USING ISOFLAVONES (57) Abstract Compositions comprising formononetin and/or one or more isoflavones selected from biochanin, genistein and daidzein, in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents are described. Also described are methods of treatment involving such compositions including the prevention and/or treatment of cardiovascular disease, the beneficial alteration of blood lipoprotein levels, or a reduction in the risk of vascular disease, or a reduction in the risk of coronary heart disease, or a reduction in the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the prevention or treatment of osteoporosis, and/or in the prevention and/or treatment of bone fracture.		

CARDIOVASCULAR AND BONE TREATMENT USING ISOFLAVONES

Field of the Invention

The present invention relates to the treatment and/or prevention of cardiovascular diseases and osteoporosis using isoflavone compounds. More particularly it relates to compositions, uses and methods involving certain plant isoflavones, and even more particularly to compositions with high formononetin content, in the prevention and/or treatment of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the prevention or treatment of osteoporosis, and/or in the prevention and/or treatment of bone fractures.

Background of the Invention

Note: References are collected at the end of the description.

Cardiovascular disease and osteoporosis have emerged as major community health issues in Western communities that are experiencing increasing longevity and in non-Western communities that are progressively westernising their lifestyles, particularly diet. Current therapeutic and preventative options for both diseases are less than satisfactory, with current options either targeting specific symptoms and failing to address the underlying pathogenic mechanisms or being associated with dose-limiting undesirable side-effects. There is an urgent need to develop safer, more effective therapies that are directed at the underlying biological events that cause cardiovascular disease and osteoporosis and which could be used both to treat existing disease states and to prevent the onset of disease, and which could be used on a long-term basis without adverse consequences.

The primary cause of cardiovascular disease is a disease of artery walls known as atherosclerosis. Atherosclerosis is characterised by the deposition of fatty plaque within the walls of blood vessels and a resulting inflammatory process induced by that plaque.

The consequence of this event is a thickening of the wall with a resulting diminution of

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the internal volume of the artery lumen. This consequence has two principal outcomes – (a) restricted blood supply to an end-organ, usually the heart (causing coronary heart disease), or kidney (causing renal failure) or the brain (causing senile dementia); and (b) acute cerebral ischaemia (or ‘stroke’) due to a piece of atheromatous plaque breaking
5 free and travelling as an embolus until it lodges in a small diameter vessel resulting in injury to the area of tissue supplied by that vessel.

An important predisposing factor to the development of atherosclerosis is the level of cholesterol in the blood, or more specifically the form in which cholesterol is present in
10 the blood. Cholesterol is an important cell structural component of cells and is required by most cells on a daily basis. Cholesterol is delivered to cells via the blood by being bound to a protein known as apoprotein of which there are several different types. The combination of cholesterol and apoprotein forms a particle known as lipoprotein. Cholesterol is delivered to cells in a particle known as low density lipoprotein
15 (abbreviated to ‘LDL’) comprising a particular type of apoprotein attached to a small number of cholesterol molecules. In the tissues, the cholesterol is detached from its carrier apoprotein and used by the cell. Any excess cholesterol sits in a free form in the tissues until being collected by another type of apoprotein. This cholesterol is returned to the liver for recycling in the form of a particle known as high density lipoprotein
20 (abbreviated to ‘HDL’). In healthy individuals, the ratio of LDL to HDL is in the range of about 2:1 to 2.5:1. It is believed generally that at this ratio, excess cholesterol is unlikely to build up in the tissues. As this ratio increases, so the ability of the body to recycle excess cholesterol diminishes, leaving free cholesterol in tissues such as artery walls. Free cholesterol, particularly in artery walls, is prone to oxidation. Oxidised
25 cholesterol is highly irritable, leading to inflammation in surrounding tissues. Atheromatous plaque is a combination of accumulating oxidised cholesterol and inflammatory tissue.

An increase in the LDL:HDL ratio above 3:1 generally is thought to be associated with
30 increased risk of atherosclerosis. A large proportion of individuals in Western

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communities have a ratio of about 3:1 to 7:1. Aside from individuals with a familial predisposition to this problem, the principal causes of this imbalance are lifestyle factors and age. It is well known that an imbalance can be due to either an abnormally elevated LDL level, or an abnormally low HDL level, or both. Factors known to be associated with an elevated LDL level are mainly dietary, e.g. a diet comprising high levels of animal fat and low levels of complex carbohydrates. Factors known to be associated with a low HDL level are lack of exercise and advancing age. The age-related effect on HDL levels is a major contributor to a high LDL:HDL ratio in older people, particularly women because HDL production is related to estrogen levels in the body and estrogen levels decline in both women and men with advancing age.

While total blood cholesterol levels are thought to be a relevant risk factor for atherosclerosis, it is now generally believed that the normal total blood cholesterol range is very wide and that a more relevant risk factor is the LDL:HDL ratio. That is, in cardiovascular risk terms, the absolute levels of both lipoprotein types is subordinate to the relative proportion of LDL and HDL.

It can be seen that in a person with an abnormally high LDL:HDL ratio, a normal ratio of about 2.5:1 might be restored by a therapeutic strategy that either lowered the LDL level, or elevated the HDL level, or both. Current therapeutic options predominantly aim to lower the LDL level and three broad approaches are used. The first approach is the use of drugs that interfere with cholesterol synthesis. The so-called 'statins', for example ethyl-2-(p-chlorophenoxy)-2-methyl-propionate, reduce cholesterol levels in the blood by interrupting cholesterol biosynthesis in the liver. These drugs typically result in a decrease in blood LDL levels by between about 10-40%. The second approach is to reduce cholesterol absorption from the gut, thereby reducing the pool of cholesterol available within the body. Historically this has been through the use of binding agents, such as insoluble, high molecular weight polymers which bind to bile acids forming a complex that is excreted in the faeces. More recently, plant sterols have been found to achieve the same result. The increased faecal loss of bile acids with either material leads

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to a decrease in LDL levels, typically in the range 5-12%. The third approach involves the use of soy protein which typically reduces total cholesterol and LDL levels by about 8-12%. The mechanism of action of this material is unknown. There are a number of deficiencies with this approach focused on lowering LDL levels. The first is that the link
5 between LDL-lowering and reduced risk of atherosclerosis or cardiovascular disease is assumed, but there is no firm clinical evidence to support this assumption. The second is that most of the current therapeutics are associated with undesirable side-effects. The statin drugs are associated with a high incidence of adverse side-effects including nausea, gastrointestinal reactions such as vomiting, loose stools, dyspepsia, abdominal distress,
10 cardiovascular complications such as increased angina or cardiac arrhythmias, dermatological problems, plus various other general complications. The resin products produce adverse reactions such as gastrointestinal disturbance, constipation, aggravation of haemorrhoids, and abdominal discomfort.

15 An alternative therapeutic option is to elevate the HDL levels. This option is increasingly being regarded by the medical profession as the more desirable option for several reasons. First, because the most conclusive evidence for a clinical benefit resulting from a re-adjustment in the LDL:HDL ratio lies with the strategy of increasing the HDL level. Gordon *et al* (1989) (*Circulation* 79: 8-15) have shown that for every 1 mg/100 mg (1%)
20 rise in HDL cholesterol in the blood, the risk of death from coronary heart disease decreases by 3%. Second, because HDL appears to provide beneficial actions on the artery wall beyond that of scavenging oxidised cholesterol. Third, because in older women in particular, the primary reason for an abnormally high LDL:HDL ratio is a decline in HDL levels. The therapeutic options here are more limited compared to those
25 targeting LDL levels. The most effective therapy is steroidal estrogen such as estradiol. Estradiol or estrogen replacement therapy typically increases HDL levels by between about 15-30% in post-menopausal women, with little or no effect on LDL levels. However, estrogen replacement therapy is associated with a number of adverse cardiovascular outcomes including a predisposition to thrombogenesis, leading to
30 increased risk of blood clots and stroke. This makes estrogen replacement therapy an

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unattractive therapeutic option for older women. Also, the feminising effects of estrogen make it even more unattractive as an option for men. Another drug substance known as clofibrate will increase HDL levels in men and women by about 10% but is little used because of its adverse side-effects. Given that the most conclusive clinical evidence for a
5 beneficial effect on atherosclerosis resulting from moderation of cholesterol levels comes from elevation of HDL levels, the current inability of medicine to offer a safe, effective means of achieving this outcome remains a major challenge.

Loss of bone density, like cardiovascular disease, is emerging as a major community
10 health problem in Western communities that are experiencing increasing longevity. As with declining HDL levels with advancing age, loss of bone density appears to be associated primarily with declining estrogen levels in the body. One of the biological effects of estrogen is the stimulation of osteoblasts, those bone cells that are responsible for the production of new bone, and the down-regulation of osteoclasts, those bone cells
15 responsible for the resorption of old bone. In the presence of low estrogen levels, osteoblast activity diminishes while osteoclast activity continues, leading to reduced production of new bone to replace the removed older bone. The result is a gradual loss of bone mass. The early stage of this condition is known as osteopenia. The later stage is known as osteoporosis. In osteoporosis the density of the bones has fallen to the point
20 where they are liable to fracture.

There are two major types of bone tissue in the body – trabecular bone and cortical bone. Trabecular bone accounts for about 80% of the bone in the body and is low density, areolar bone. Trabecular bone predominates in those bones or in those parts of bones
25 which are not highly weight-bearing, such as the vertebrae, ribs, skull, wrist and ankle. Cortical accounts for the remaining 20% of bone in the body and is very dense bone. Cortical predominates in load-bearing situations such as the long bones of the limbs and the femoral neck in the hip joint.

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Bone density begins to decline normally from about middle age in both men and women and both trabecular and cortical bone are affected, although trabecular bone has a higher natural turn-over rate compared to cortical bone, and trabecular bone typically experiences a greater rate of loss of density in the early part of this osteopenic process.

5 Menopause accelerates this process in women. Near menopause, trabecular bone has about an eightfold greater rate of turnover compared to cortical bone. In peri-menopausal women, trabecular bone is lost by between about 4-8% per annum versus 2-3% per annum for cortical bone. By about the age of 60, the rates of loss in both trabecular and cortical bone approximate. This age-related effect is responsible for the phenomenon in
10 menopausal women where fractures seen between the ages of 50-60 years typically involve the vertebrae, wrist and ribs (predominantly trabecular bone) and over the age of 60 years typically involve the hip and long bones (predominantly cortical bone). Hip and femur fracture are the most serious of the various bone fractures, requiring extended hospitalisation and usually extensive surgery. About one-third of older women who
15 fracture their hip die within 12 months of the fracture because of related complications.

There are various therapeutic options for the treatment or prevention of osteoporosis. Steroidal estrogens such as estradiol or synthetic derivatives such as raloxifene are well known and widely used for these purposes. These compounds function through a
20 combination of promotion of bone deposition and reduction in bone resorption. However, their effect is seen principally on trabecular bone and they have little or no effect on cortical bone. The result of this selective action is that they may protect against fracture of bones such as the wrist, ribs and vertebrae, but provide little or no protection against the more serious hip and femur fractures. A class of compounds known as
25 bisphosphonates also enjoy common usage. These compounds act by decreasing bone resorption, and while affecting both trabecular and cortical bone, like the estrogens, their effect is predominantly directed towards trabecular bone. Other therapies include calcitonin, which decreases the rate of bone resorption and ipriflavone which inhibits bone resorption and increases osteoblast function. All of these drugs are associated with
30 undesirable side-effects. Given that effective therapy or prevention of bone fractures

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requires long-term therapy of between 10-30 years, safety and tolerability are key issues for patients and all the above therapies enjoy poor patient compliance because of their low safety profiles. There is an urgent need to develop therapies that are particularly directed to protection of cortical bone and which have a high safety and tolerability profile so as to encourage long-term usage.

Some interest has been shown in recent years in plant compounds known as isoflavones, in particular those with estrogenic function such as genistein and daidzein and their methyl esters, biochanin and formononetin. In part this interest stems from the epidemiological observations that cardiovascular diseases and osteoporosis are less common in communities whose diets are rich in isoflavones. In part, it also stems from their estrogenic function and the likelihood that they could mimic the health benefits of estradiol, in particular in the positive cardiovascular health benefits and bone density-raising effects of estradiol. Most scientific interest has focused on genistein and daidzein as these are the strongest estrogen agonists of the four isoflavones. Genistein and daidzein are reported to have an estrogenic potency approximately 0.1 % that of estradiol, while formononetin and biochanin are about 10-100x weaker than that.

The literature is minimal in respect to osteoporosis and isoflavone studies. US Patent 5424331 discloses the use of genistein and daidzein as components of an extensive mixture of specified compounds in the prevention and/or treatment of osteoporosis in humans. However, that patent does not teach the beneficial use of the isoflavones formononetin and biochanin, or the effect of a particular isoflavone ratio, or the beneficial effect of isoflavones alone, or the relative effect of the isoflavones either alone or in combination with other materials on trabecular or cortical bone.

There is experimental evidence that genistein has a beneficial effect on bone. Genistein is reported to stimulate bone formation (Fanti, 1998) and to depress osteoclast activity (US patent 5,506,211 Barnes, S and Blair, HC: Genistein for use in inhibiting osteoclasts). Low doses of genistein reportedly increased both cortical and trabecular bone density in

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rats (Anderson, 1998). While the group of isoflavones, genistein, daidzein, formononetin and biochanin are known to share some biological properties, it is also well known that they vary considerably in their biological potencies. Thus there is no understanding of the effect of formononetin, biochanin or daidzein on bone biology.

5

The only reported clinical study involving isoflavones and osteoporosis involved 66 postmenopausal women in a placebo-controlled study who were treated for 6 months with a soy product containing either 'moderate' or 'high' isoflavone levels (Potter S.M. *et al* "Soy protein and isoflavones: their effects on blood lipids and bone density in
10 postmenopausal women" *American Journal of Clinical Nutrition* 1998: 68(suppl) 1375S-1379S). It is well known that soy contains daidzein and genistein approximately in a ratio of 1:2 and does not contain appreciable levels of formononetin or biochanin. The outcome of this study was that the 'high' isoflavone material resulted in a 2% increase in bone mineral content and density of lumbar spine but had no effect on bone mineral
15 content or density of the femur. The implication from these results is that daidzein and genistein have a modest effect on trabecular bone but no effect on cortical bone.

The literature is somewhat clearer on isoflavones and lipoprotein levels. There are a number of animal and human studies where whole foodstuffs such as soya or other
20 legumes or even relatively crude extracts of soya or other legumes have been fed to recipients and lipoprotein levels monitored. At best these data are highly equivocal and variable. But more importantly, the use of such crude preparations entails the concomitant use of so many plant components including many such as saponins and sterols that are known to have modulating effects on cholesterol metabolism, that it is not
25 possible for even those skilled in the art to draw any relationship between isoflavones and blood lipoprotein levels.

The most telling evidence comes from those studies where supplements are highly enriched for isoflavones and where there no other dietary variation have been used.
30 Three studies using soy extracts enriched for the isoflavones genistein and daidzein have

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been reported. Two studies failed to find any significant effects of the dietary supplementation on LDL or HDL levels (Nestel *et al* (1997) "Soy Isoflavones Improve Systemic Arterial Compliance but Not Plasma Lipids in Menopausal and Perimenopausal Women" *Arteriosclerosis, Thrombosis and Vascular Biology* Biol 17: 3392-3398) and
5 Hodgson *et al* (1998) "Supplementation with Isoflavonoid Phytoestrogens Does not Alter Serum Lipid Concentrations: A Randomised Controlled Trial in Humans" *Journal of Nutrition* 128, 728-332). In a third study (Potter S.M. *et al* as above), 6 months' therapy with a soy powder in hypercholesterolemic, post-menopausal women produced a mean 4.3% increase in HDL levels and a mean 8% decrease in LDL levels. The first two
10 studies would be considered generally to be a more reliable indicator of the lack of effect of soy isoflavones on lipoprotein levels given that the isoflavones were added in a highly concentrated form and necessitated little dietary adjustment. In the third study, the isoflavones were delivered via a soy powder which apart from containing a wide variety of soy components such as saponins and sterols with known cholesterol-modifying
15 properties, also is well known to modify dietary habits through the weight of protein present in the soy product.

US Patent No. 5855892 (Potter) describes a method of altering the concentration of cholesterol constituents in human blood using the isoflavone daidzein. Potter describes
20 the use of soy protein and the isoflavones genistein, daidzein, glycitein and their respective glycosides. A 5.2% increase in HDL-cholesterol concentration was reported in subjects receiving the soy protein/isoflavone composition. It is unclear what the active agent in the compositions is, although the applicants believe it is the soy protein constituent which may be providing the very modest increase in HDL levels.

25

Further work by Nestel *et al* (1999 "Isoflavones From Red Clover Improves Systemic Arterial Compliance but Not Plasma Lipids in Menopausal Women" *Journal of Clinical Endocrinology and Metabolism* 84: 895-898) has shown that dosage with isoflavones from red clover, comprising biochanin, formononetin, daidzein and genistein in the
30 approximate ratio 1.8:1.2:0.2:0.1 also have no effect on plasma lipids. Another study using

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a similar supplement of isoflavones from red clover and conducted in normocholesterolemic, premenopausal women found no statistically significant effect of isoflavone supplementation on LDL or HDL levels although there was a slight increase in the HDL₃ sub-fraction (Samman S, *et al* "The effect of supplementation with isoflavones on plasma lipids and oxidisability of low density lipoprotein in premenopausal women" *Atherosclerosis* 147, 277-283 (1999).

A reasonable summary of the known art would be that isoflavones from the group genistein, daidzein, formononetin and biochanin either singly or in varying combinations have little or no effect on blood lipoprotein levels.

Summary of the Invention

It has been surprisingly found by the inventors that compositions comprising high proportions of formononetin relative to one or more isoflavones selected from biochanin, genistein and daidzein, in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents, are useful in the prevention and/or treatment of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as to prevent or treat osteoporosis, and/or in the prevention and/or treatment of bone fracture.

These particular health benefits found with a composition containing such a high formononetin content is highly unexpected and surprising for two principal reasons. First, because it is generally believed that any beneficial effect of isoflavones on the cardiovascular system or bone is associated with their estrogenic effect and formononetin displays the weakest estrogenic function of the group of isoflavones comprising genistein, daidzein, formononetin and biochanin. Second, because it also is assumed generally that the human body effectively demethylates formononetin to daidzein, meaning that formononetin should have equivalent function to daidzein.

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In accordance with a first aspect of this invention there is provided a composition comprising formononetin and one or more isoflavones selected from biochanin, genistein and daidzein, in a therapeutically effective ratio of formononetin to said isoflavone(s) of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents.

Formononetin and one or more isoflavones selected from biochanin, genistein and daidzein are preferably provided in the form of extracts from chickpea, clover, or other plant sources high in formononetin context. The extracts may be prepared by water/organic solvent extracts of legume plants, such isoflavone extractive procedure being well known in the art. Alternatively, isoflavones may be produced by established synthetic techniques as are well known in the art. Formononetin may be in association with one or two or three isoflavones selected from biochanin, genistein and daidzein. Preferably, the formononetin is present in association with biochanin, free of genistein and daidzein or with trace levels or low levels of these components, such as from 0.1% to 5% w/w of isoflavone content.

In accordance with another aspect of this invention there is provided use of formononetin and one or more isoflavones selected from biochanin, genistein and daidzein in the ratio of 15:1 to 2:1 for the manufacture of a medicament for the treatment and/or prevention of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the treatment or prevention of osteoporosis, and/or in the prevention and/or treatment of fracture.

In another aspect of the invention there is provided a method for the prevention and/or treatment of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in

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the treatment or prevention osteoporosis, and/or in the prevention and/or treatment of bone fracture, which comprises administering to a human subject a composition comprising formononetin and one or more of biochanin, genistein and daidzein in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries, and/or diluents.

Detailed Description

Compositions of the present invention comprise formononetin and one or more isoflavones selected from biochanin, genistein and daidzein, in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents. Formononetin may be present in association with one or more of biochanin, genistein and daidzein. Where formononetin is in association with a single isoflavone, that isoflavone is preferably biochanin, although having said this, biochanin may be replaced by genistein or daidzein. Where two of biochanin and genistein, biochanin and daidzein or genistein and daidzein are present in addition to formononetin, they may be present in equal amounts on a weight to weight basis, or from 5% through to 95% on a weight to weight basis of a first isoflavone, with a corresponding amount of the second isoflavone. Where the composition comprises formononetin, and biochanin, genistein and daidzein, wherein the ratio of formononetin to said other isoflavones is 15:1 to 2:1, the biochanin, genistein and daidzein may be present in equal amounts on a weight to weight basis, or alternatively in varying amounts, the varying proportions of these isoflavones not being important to the invention. Thus, one "unit" of a combination of biochanin, genistein and daidzein may comprise from 0.1 to 0.99 units biochanin, from 0.1 to 0.99 units daidzein, and from 0.1 to 0.99 units daidzein, giving an "other" (non-formononetin) isoflavone content of one unit. What is particularly significant to the invention is the high formononetin content with regard to other isoflavones, particularly biochanin, genistein and/or daidzein.

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The effect of this high formononetin ratio is to produce an unexpectedly large increase in HDL levels in the blood, an effect known to be highly beneficial in protecting against atherosclerosis and coronary heart disease. Similarly, the findings as shown hereafter that the effect of this composition detailed in this invention has a particular and dramatic effect on cortical bone density is both unexpected and indicative of a significant clinical benefit on the initiation and progression of osteoporosis and the resulting risk of bone fracture, particularly of the hip joint, humerus, femur, radius and ulna. The magnitude of these biological effects and resulting clinical outcomes obtained with the composition detailed in this invention is of a magnitude so greater than that known to be obtained with isoflavones generally to indicate that it is a function specifically of this particular isoflavone ratio.

Formononetin and one or more isoflavones selected from biochanin, genistein and daidzein are preferably provided in the form of extracts from chickpea or clover which high in formononetin context. The extracts are preferably water/organic solvent extracts, this isoflavone extractive procedure being well known in the art.

Clover, for example red clover, is a preferred source of formononetin and said other isoflavones. Clovers which may be used include red clover (*T. pratense*) or subterranean clover (*T. subterranean*). Many types of red and other forms of clovers are known, and being developed. These legumes may be used in the present invention. The aforementioned isoflavones are preferably prepared by extracting the leguminous material with a water/organic solvent.

Collected plant material may be comminuted or chopped into smaller pieces, partially comminuted or chopped into smaller pieces, or contacted without any pre-treatment with generally water and an organic solvent, such as a water miscible organic solvent. The ratio of water to organic solvent may be generally in the range of 1:10 to 10:1 and may for example comprise equal proportions of water and solvent or from 1% to 30% (v/v) organic solvent. Any organic solvent or a mixture of such solvents may be used. The

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organic solvent may preferably be a C2-10, more preferably a C1-4 organic solvent (such as methanol, chloroform, ethanol, propanol, propylene glycol, erythrite, butanol, butanediol, acetonitrile, ethylene glycol, ethyl acetate, glycidol, glycerol dihydroxyacetone or acetone). The extract in this regard may be prepared by exposing
5 the plant material to the water/solvent mix. Optionally the mixture may include an enzyme which cleaves isoflavone glycosides to the aglycone form. The mixture may be vigorously agitated so as to form an emulsion. The temperature of the mix may range, for example, from an ambient temperature to boiling temperature. Exposure time may be between one hour to several weeks. One convenient extraction period is twenty-four
10 hours at 90°C. The extract may be separated from undissolved plant material and the organic solvent removed, such as by distillation, rotary evaporation, or other standard procedures for solvent removal. The resultant extract containing water soluble and non-water soluble components may be dried to give an isoflavone-containing extract, which may be formulated with one or more pharmaceutically acceptable carriers, excipients
15 and/or auxiliaries.

The extract following distillation contains a small amount of oil which includes isoflavones in their aglycone form (referred to herein as isoflavones). This isoflavone enriched oil, may be subject to HPLC to adjust the isoflavone ratios, or, if at the desired,
20 isoflavone ratio may be dried, for example in the presence of silica, and be formulated with one or more carriers, excipients and/or auxiliaries to give an isoflavone containing extract. Alternatively, isoflavones may be further concentrated by addition to the oil of a non-water soluble organic solvent such as hexane, heptane, octane acetone or a mixture of one or more of such solvents. One example is 80% hexane, 20% acetone w/w having
25 high solubility for oils but low solubility for isoflavones. The oil readily partitions into the organic solvent, and an enriched isoflavone containing extract falls out of solution. The recovered extract may be dried, for example in an oven at 50°C to about 120°C, and formulated with one or more pharmaceutically acceptable carriers, excipients and/or auxiliaries. The ratio of isoflavones, from legume extracts, particularly the high content
30 of formononetin to other isoflavones is readily obtained and adjusted, for example by use

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of clovers of high formononetin content, concentration using various solvents as described above, HPLC fractionation, and the like.

5 Clover, as the preferred legume source, is readily extract with water/organic solvents. A single source of clover may be used, or a combination of one or more different clovers and/or chickpeas employed.

Formononetin and the other isoflavones referred to herein may be synthetically produced according to methods well known in the art. See for example Kagal et al, Tetrahedron
10 Letters 1962, 593; Mahal et al, *J Chem Soc* 1934, 1769; Wahala et al, *Proc Soc Exp Biol Med* 208, 18(1995) at 27-32.

The compositions according to the present invention may include one or more pharmaceutically acceptable carriers. Carriers are selected so as to be acceptable in the
15 sense of being ingredients in the composition and must not be deleterious to the patient. The carriers may be solid or a liquid, or both, and may be formulated with an extract containing the isoflavones at the desired ratios as a unit-dose, for example a tablet, which may contain from 0.5% to 80% by weight of extract or up to 100% by weight to extract. Compositions may be prepared by any of the well known techniques of pharmacy, for
20 example admixing the extract, optionally including excipients, diluents (for example, water) and auxiliaries as are well known in the pharmaceutical field.

The compositions according to the invention may include one or more agents, such as vitamins (for example, Vitamin A, Vitamin B group, Vitamin C, Vitamin D, Vitamin E
25 and Vitamin K), and minerals (for example, magnesium, iron, zinc, calcium and manganese in the form of pharmaceutically acceptable salts).

The compositions of the invention include those suitable for oral, rectal, optical, buccal (for example, sublingual), parenteral (for example, subcutaneous, intramuscular,
30 intradermal and intravenous) and transdermal administration. The most suitable route in

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any given case will depend on the nature and severity of the condition being treated and the state of the patient.

Compositions suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a pre-determined amount of the extract; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. Such compositions may be prepared by any suitable method of pharmacy which includes the step of bringing into association the active compound and one or more suitable carriers (which may contain one or more accessory ingredients as noted above). In general the compositions of the invention are prepared by uniformly and intimately admixing the extract with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by comprising or moulding a powder or granules containing the extract, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, the extracts in the form of a powder or granules optionally mixed with a binder, lubricant, inert diluents, and/or surface active/dispersing agent(s). Moulded tablets may be made by moulding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

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Suitable carriers may be fillers, such as sugars, for example lactose, saccharose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example, tricalcium phosphate or calcium hydrogen phosphate, and also binders, such as starch pastes using, for example, corn, wheat, rice or potato starch, gelatin, tragacanth, methylcellulose and/or polyvinylpyrrolidone, and, if desired, disintegrators, such as the above-mentioned starches, also carboxymethyl starch, cross linked polyvinylpyrrolidone, agar or algin acid or a salt thereof, such as sodium alginate. Excipients may be flow conditioners and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable, optionally enteric, coatings, there being used, *inter alia*,

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concentrated sugar solutions which may comprise gum Arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents or solvent mixtures, or, for the preparation of enteric coatings, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate. Dyes or pigments may be added to the tablets or dragee coatings, for example, for identification purposes or to indicate different doses of active ingredients.

Other orally administrable pharmaceutical compositions are dry-filled capsules made, for example, of gelatine, and soft, sealed capsules made of gelatine and a plasticiser, such as glycerol or sorbitol. The dry-filled capsules may comprise the extracts in the form of granules, for example, in admixture with fillers, such as lactose, binders, such as starches, and/or glicants, such as talc or magnesium stearate, and, where appropriate, stabilisers. In soft capsules, the extract is preferably dissolved or suspended in liquids, such as fatty oils, paraffin oil or liquid polyethylene glycols, to which stabilisers may also be added.

Formulations suitable for buccal (sublingual) administration include lozenges comprising the extracts in a flavoured base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound in an inert base such as gelatine and glycerin or sucrose and acacia.

Compositions of the present invention suitable for parenteral administration conveniently comprise sterile aqueous preparations of the extracts, which preparations are preferably isotonic with the blood of the intended recipient. These preparations are preferably administered intravenously, although administration may also be effected by means of subcutaneous, intramuscular, or intradermal injection. Suitable compositions include water soluble extracts and also suspensions of the active ingredient, such as corresponding oily injection suspensions, there being used suitable lipophilic solvents or vehicles, such as fatty oils, for example sesame oil, or synthetic fatty acid esters, for

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example ethyl oleate or triglycerides, or aqueous injection suspensions comprising viscosity-increasing substances, for example sodium carboxymethylcellulose, sorbitol and/or dextran, and, where appropriate, also stabilisers. As an example, compositions may conveniently be prepared by admixing the extracts with water or a glycine buffer and rendering the resulting solution sterile and isotonic with the blood. Injectable formulations according to the invention may contain from 0.1% to 60% w/v of the extract and may, for example, be administered at a rate of 0.1 ml/minute/kg.

Formulations suitable for rectal administration are preferably presented as unit dose suppositories. These may be prepared by admixing the extracts with one or more conventional solid carriers, for example cocoa butter, and then shaping the resulting mixture.

Compositions suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches may contain the extracts in an optionally buffered aqueous solution.

Compositions suitable for transdermal administration may also be delivered by iontophoresis (see, for example, Pharmaceutical Research 3 (6): 318 (1986)) and typically take the form of an optionally buffered aqueous solution of the extracts. Such compositions may, for example, contain citrate or bis/tris buffer (pH 6) or ethanol/water, with for example 0.05% to 30% w/w extract.

Compositions may be prepared in a manner, and in a form/amount as is conventionally practised. See, for example, Goodman & Gillman, The Pharmacological Basis of Therapeutics (7th Edition, 1985) and Remington's Pharmaceutical Science (Mack Publishing Company, 10th Edition), both of which are incorporated herein by reference. Compositions may contain, for example, from 0.1 mg to 2 g isoflavones, such as 0.1 mg

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to 200 mg, more particularly 15 mg to 50 mg isoflavones, the ratios on a w/w basis between the isoflavones being as described above.

The compositions of the invention may also be administered to a human in a dietary supplement form. Dietary supplements incorporating the active composition can be prepared by adding the composition to a food in the process of preparing the food. Any food may be used including, but not limited thereto, meats such as ground meats, emulsified meats and marinated meats; beverages such as nutritional beverages, sports beverages, protein fortified beverages, juices, milk, milk alternatives, and weight loss beverages; cheeses such as hard and soft cheeses, cream cheese, and cottage cheese; frozen desserts such as ice cream, ice milk, low fat frozen desserts, and non-dairy frozen desserts; yogurts; soups; puddings; bakery products; salad dressings; and dips and spreads such as mayonnaise, margarine, butter, butter substitute, and other fat containing spreads. The composition is added to the food in an amount selected to deliver a desired dose of the composition to the consumer of the food.

The isoflavones as referred to above may be in the form of a powder, a slurry, in aqueous solution (for example, containing a small amount of oil), particulate form, or dissolved in an organic solvent (such as methanol, ethanol, ethyl acetate or dimethyl sulphoxide).

An effective amount of the compositions of the present invention is administered to a human subject. The actual dosage levels will depend upon a number of factors, such as specific mode of administration, the condition being treated, the condition of the patient and the judgement of the health care giver. Examples of dosages of isoflavones are about 0.1 mg to about 200 mg per day, such as in the order of 1.5 mg/kg (body weight)/day. A convenient dosage form contains about 25 mg to 50mg isoflavone as described herein.

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The composition of the present invention comprises formononetin as the principal isoflavone. In the context of this invention, it has been found that the preferred ratio of formononetin to the other three main isoflavones, genistein, daidzein and biochanin embraces the various naturally-occurring forms of isoflavones including their aglycone, glycoside, acetyl or malonyl forms.

In accordance with another aspect of this invention there is provided use of formononetin and one or more isoflavones selected from biochanin, genistein and daidzein in the ratio of 15:1 to 2:1 for the manufacture of a medicament for the prevention and/or treatment of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels or to reduce the risk of coronary heart disease, or in the beneficial alteration or maintenance of bone density such as in the prevention or treatment of osteoporosis, and/or to prevent and/or treat bone fracture. Formononetin and one or more of biochanin, genistein and daidzein, are provided in a ratio of formononetin to the other isoflavones, whether alone or in combination, in a ratio of 15:1 to 2:1, preferably 10:1 to 5:1. A composition formed therefrom may then be administered to humans.

In another aspect of this invention there is provided a method for the treatment and/or prevention of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the treatment or prevention osteoporosis, and/or in the prevention and/or treatment of bone fracture, which comprises administering to a human subject a composition comprising formononetin and one or more of biochanin, genistein and daidzein in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries, and/or diluents.

A further method aspect of this invention is a method for the beneficial alteration of blood lipoprotein levels. In this aspect HDL levels may be increased and/or LDL levels

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may be decreased. Accordingly there is provided a method of increasing HDL levels in a subject. In another aspect there is provided a method of reducing LDL levels in a subject.

- 5 The method aspect of this invention may also extend to a method to decrease the propensity of thrombogenic events in humans.

In a further method aspect of this invention there is provided a method to reduce the risk of vascular disease, coronary heart disease and/or arteriosclerosis in a human.

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In a further method aspect of this invention there is provided a method for the beneficial alteration or maintenance of bone density such as in the treatment or prevention of osteoporosis.

- 15 In a still further method aspect there is provided a method of preventing and/or treating fractures (including accelerating healing) involving bone with predominant cortical bone tissue, such as those involving the femoral neck, femur, humerus, radius, ulna and tibia.

- Each of these above methods involves administering to a human subject a composition
20 comprising formononetin and one or more of biochanin, genistein and daidzein in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents. The subjects being treated may be post-menopausal women who are normocholesterolemic or hypercholesterolemic, women who are artherosclerotic, post-
25 menopausal women with low HDL, and males who are hypercholesterolemic or normocholesterolemic, and/or artherosclerotic.

- Oral administration of a solid dosage form such as a tablet or capsule is preferred. One or more daily doses is a standard dosing regime. Administration may continue until, for
30 example, lipid levels in the blood are moved to the appropriate levels. However, for

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maximal benefits on lipid ratios, or prevention of cardiovascular disease, or reduction in the risk of coronary heart disease, or reduction in the risk of arteriosclerosis, or in the beneficial alteration of bone density such as prevention of osteoporosis, and/or in the prevention of bone fractures, administration may be long term, such as for one or more
5 years.

As mentioned above, the ratio of formononetin to other isoflavones as used in the compositions, methods and uses of this invention produces surprising and most advantageous effects in relation to HDL increase (notwithstanding total cholesterol
10 increase), and cortical bone mass increase.

This invention will now be described with reference to the following non-limiting examples.

15 Example 1

A tablet containing an extract enriched for isoflavones was prepared by methods well known in the fields of pharmaceutical and botanical chemistry. Specifically, the isoflavones are extracted from a legume such as red clover using a standard water/alcohol extract procedure (as described in the patent PCT/AU9800305) and the
20 extract formed into a tablet using standard methods. More specifically, the type of red clover used should contain a mixture of formononetin, biochanin, daidzein and genistein.

Briefly, red clover leaves are harvested and macerated so as to induce enzymatic degradation of isoflavones from their glycosidic form to their aglycosidic form. After
25 standing at ambient temperature for 2 hours, the plant material is snap-frozen by exposure to liquid nitrogen. The material can be stored in this form for up to several years. For extraction, the frozen material is crushed to a fine powder, thawed and placed in a fine gauze bag that is immersed in a solution of 60% ethanol in water. Extraction is carried out at 60°C for twenty four hours. The supernatant is separated
30 from the undissolved plant material, and the solvent removed by distillation. The aqueous

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phase containing the isoflavones is extracted again with an organic solvent (either petroleum ether or hexane or acetyl acetate) to remove oils and other polar compounds. The solvent then is removed by distillation and the aqueous phase taken to near-dryness by rotary evaporation. This generates a concentrated extract comprising about 25% isoflavones on a dry weight basis.

This process essentially extracts the isoflavones on a non-preferential basis so that the original ratio of the four isoflavones in the plant is essentially preserved in the final extract. In this example, a strain of red clover was selected that contains the four isoflavones in the approximate ratio (as detected by thin layer chromatography) of 45% biochanin, 40% formononetin, 8% daidzein and 7% genistein.

The dried isoflavone extract was mixed with standard excipients such as methylcellulose to form a 400 mg tablet containing 160 mg clover extract and more specifically, 40 mg of isoflavones comprising 18 mg biochanin, 16 mg formononetin, 3 mg daidzein and 3 mg genistein.

Example 2

A tablet is made according to the procedure detailed in Example 1, but in this case a strain of red clover is selected that has a high formononetin content. The strain selected has a ratio of 82% formononetin, 12% biochanin, 3% daidzein and 3% genistein. After solvent extraction as detailed in Example 1, the dried isoflavone extract has approximately the same isoflavone ratio as in the starting plant material.

A 200 mg tablet is formulated using 100 mg of the dried plant extract containing 25 mg of isoflavones comprising approximately 20 mg formononetin, 3 mg biochanin, 1 mg daidzein and 1 mg genistein.

Example 3

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Thirty-six post-menopausal normocholesterolemic women were recruited into a double-blind clinical trial and randomly allotted to one of three treatment arms – (a) placebo tablet, (b) 1x isoflavone tablet daily, or (c) 3x isoflavone tablets daily. The tablets used were those prepared as in Example 1. Treatment continued for 3 months. Blood was collected both at the commencement and completion of the study and analysed for total cholesterol, LDL and HDL levels and clotting factors. No significant changes were found in any of these parameters in any of the treatment arms over the course of the study.

10 Example 4

Fifty post-menopausal, normocholesterolemic women were recruited into a single-blind clinical trial and randomly allocated to three treatment groups. All three groups received a monthly run-in using a daily placebo tablet. They then received an isoflavone supplement enriched for formononetin in the form of a tablet as prepared in Example 2. Three doses were used – either 25 mg, 50 mg or 75 mg isoflavones daily. The principal outcomes monitored were total cholesterol, HDL and non-HDL (mainly LDL) levels and bone density of the proximal forearm (predominantly cortical bone) and distal forearm (predominantly trabecular bone). The results are summarised as follows as % change after 6 months' therapy from baseline.

Isoflavone concentration	Total cholesterol	HDL	LDL	Apoprotein B	Proximal forearm	Distal forearm
25 mg	4.85	16.5	1.18	-10.59	2.9	-1.4
50 mg	6.19	28.6	6.99	-9.72	4.1	-1.1
75 mg	5.87	15.75	6.26	-12.15	2.99	1.7

It can be seen that all three doses of this particular ratio of isoflavones resulted in a variety of statistically significant and clinically significant changes. Total cholesterol levels rose slightly (7%) in all three groups and this was attributable to the dramatic rise in HDL levels. LDL levels were not significantly affected, but HDL levels rose by as much as mean 28% in the 50 mg isoflavone group, an entirely unexpected outcome given

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the lack of effect on HDL levels observed with other isoflavone studies. A significant decline in blood levels of apoprotein B also was achieved, and again this was entirely unexpected and points to a significant clinical benefit for these women in terms of their risk factors for cardiovascular disease.

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Women in the treatment groups, particularly the 50 mg treatment group, also showed a highly significant and positive effect on cortical bone density (proximal forearm) in the first six months (4.1% increase). There was no observed effect on trabecular bone (distal forearm), indicating that this particular isoflavone ratio is having a highly specific effect on cortical bone. Again this is an entirely unexpected outcome given the lack of any previous description of any product that shows specific increase on cortical bone with no effect on trabecular bone.

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Example 5

Based on the results of Example 4, a 50 mg isoflavone-containing tablet prepared according to Example 2 was tested to see if a particularly refractile treatment group, namely women with high LDL and low HDL could benefit from the treatment of the present invention. This particular grouping of patients are regarded as metabolising cholesterol in a manner different from normocholesterolemic subjects.

20

The study design was five weeks on the active composition or placebo, and then a cross-over to alternative treatment (active-groups switched to placebo and placebo groups switched to active) for another five weeks. Then all subjects remained on the active for another 12 weeks. Results shown in the table below present data for the "short-active" treatment group which comprise women on active for five weeks, and the data in the "extended-active" column were for women on active for 17 weeks (i.e. 5 plus 12 weeks).

25

	Placebo	Short-active	Extended-active
HDL cholesterol (mol)	1.38	1.34	1.34

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LDL cholesterol (mol)	5.59	5.33	5.08
Number of subjects	22	22	11

This study unexpectedly shows that women with high HDL and low LDL at entry to the study exhibited a significant reduction in LDL, in the order of 10%. This finding correlates with or is somewhat better than current best practice pharmaceutical agents such as the statins. No examples exist in the literature of the use of isoflavones in women with this type of lipoprotein profile. Accordingly, the compounds of the present invention offer treatment for this particularly refractile patient group.

Example 6

25 mg, 50 mg and 75 mg tablets were prepared with the following excipients to form a tablet of total weight 550 mg. The isoflavones were mixed with an acacia gum carrier, then added to a tableting formulation containing mixed tocopherols, cellulose microcrystalline, calcium hydrogen phosphate, soy polysaccharide, magnesium stearate and silica-colloidal anhydrous.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Patents:

PCT Patent No. PCT/AU98/00305 Kelly G. et al: Preparation of isoflavones from legumes.

- 5 US Patent No. 5855892 Potter S.M., Henley E.C., Waggle D.H.: *Method for decreasing LDL-cholesterol concentration and increasing HDL-cholesterol concentration in the blood to reduce the risk of atherosclerosis and vascular disease.*

US Patent 5506211 Barnes S. and Blair H.C.: *Genistein for use in inhibiting osteoclasts.*

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US Patent 5,424,331 6/1995 Shylankevich M. 514-456 *Pharmaceutical compositions and dietary soybean food products for the prevention of osteoporosis.*

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CLAIMS

1. A composition comprising formononetin and one or more isoflavones selected from biochanin, genistein and daidzein, in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents.
2. A composition according to claim 1 wherein the ratio of formononetin to said isoflavones is from 10:1 to 2:1.
3. A composition according to claim 1 comprising formononetin and biochanin.
4. A composition according to claim 1 in the form of a solid dosage unit.
5. A composition according to claim 4 in the form of a tablet, capsule, granular preparation, buccal delivery vehicle or suppository.
6. A composition according to claim 1 wherein said formononetin and biochanin comprise an extract of chickpea or clover.
7. A composition according to claim 6 wherein said clover is selected from red clover (*T. pratense*), subterranean clover (*T. subterranean*) or white clover (*T. repens*).
8. A composition according to claim 7 wherein said clover is red clover.
9. A composition according to claim 1 for the prevention and/or treatment of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density

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CLAIMS

1. A composition comprising formononetin and one or more isoflavones selected from biochanin, genistein and daidzein, in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries and/or diluents.
2. A composition according to claim 1 wherein the ratio of formononetin to said isoflavones is from 10:1 to 2:1.
3. A composition according to claim 1 comprising formononetin and biochanin.
4. A composition according to claim 1 in the form of a solid dosage unit.
5. A composition according to claim 4 in the form of a tablet, capsule, granular preparation, buccal delivery vehicle or suppository.
6. A composition according to claim 1 wherein said formononetin and biochanin comprise an extract of chickpea or clover.
7. A composition according to claim 6 wherein said clover is selected from red clover (*T. pratense*), subterranean clover (*T. subterranean*) or white clover (*T. repens*).
8. A composition according to claim 7 wherein said clover is red clover.
9. A composition according to claim 1 for the prevention and/or treatment of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density

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such as in the prevention or treatment of osteoporosis, and/or in the prevention and/or treatment of bone fracture.

10. A composition according to claim 1 which additionally includes one or more
5 vitamins.
11. A composition according to claim 10 wherein said vitamins are selected from
one or more of vitamin C, vitamin D, vitamin E, vitamin K, vitamin A, and
vitamin B.
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12. A composition according to claim 1 which additionally includes a calcium
containing compound (0.5 g to 2 g).
13. A composition according to claim 1 which is incorporate into a beverage.
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14. A composition according to claim 13 wherein the beverage is selected from a
nutritional beverage, sports beverage, juice, milk or milk alternative.
15. A composition according to claim 1 which is incorporated into a food.
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16. A composition according to claim 15 where the food is yogurt, a food bar, a
spread, hard cheese, soft cheese, cream cheese or cottage cheese.
17. A composition according to claim 1 in the form of a pharmaceutical composition.
25
18. A composition according to claim 17 which is in the form of a pill, tablet,
capsule, suppository, dragee or sublingual dosage form.
19. Use of formononetin and one or more isoflavones selected from biochanin,
30 genistein and daidzein in the ratio of 15:1 to 2:1 for the manufacture of a

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medicament for the treatment and/or prevention of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to decrease the propensity of thrombogenic events in humans, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the prevention and/or treatment of osteoporosis, and/or in the prevention and/or treatment of fracture.

20. Use according to claim 13, which includes providing formononetin and biochanin in a ratio of 15:1 to 2:1, and forming a composition for administration to humans in admixture with one or more carriers, excipients, auxiliaries or diluents.

21. A method for the treatment and/or prevention of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the treatment or prevention of osteoporosis, or in the prevention and/or treatment of bone fracture, which comprises administering to a human subject a composition comprising formononetin and one or more of biochanin, genistein and daidzein in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries, and/or diluents.

22. A method according to claim 21 which is a method for the beneficial alteration of blood lipid protein levels.

23. A method according to claim 22 which increases the concentration of high density lipoprotein in the blood of said human.

24. A method according to claim 23 which decreases the concentration of low density lipoprotein in the blood of said human.

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medicament for the treatment and/or prevention of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to decrease the propensity of thrombogenic events in humans, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the prevention and/or treatment of osteoporosis, and/or in the prevention and/or treatment of fracture.

20. Use according to claim 13, which includes providing formononetin and biochanin in a ratio of 15:1 to 2:1, and forming a composition for administration to humans in admixture with one or more carriers, excipients, auxiliaries or diluents.

21. A method for the treatment and/or prevention of cardiovascular disease, or the beneficial alteration of blood lipoprotein levels, or to reduce the risk of coronary heart disease, or to reduce the risk of arteriosclerosis, or in the beneficial alteration or maintenance of bone density such as in the treatment or prevention osteoporosis, or in the prevention and/or treatment of bone fracture, which comprises administering to a human subject a composition comprising formononetin and one or more of biochanin, genistein and daidzein in a therapeutically effective ratio of formononetin to said isoflavones of 15:1 to 2:1, optionally in association with one or more carriers, excipients, auxiliaries, and/or diluents.

22. A method according to claim 21 which is a method for the beneficial alteration of blood lipid protein levels.

23. A method according to claim 22 which increases the concentration of high density lipoprotein in the blood of said human.

24. A method according to claim 23 which decreases the concentration of low density lipoprotein in the blood of said human.

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25. A method according to claim 21 to reduce the risk of vascular disease.
26. A method according to claim 21 to reduce the risk of coronary heart disease.
- 5 27. A method according to claim 21 to reduce the risk of arteriosclerosis.
28. A method according to claim 21 for the treatment or prevention of osteoporosis.
- 10 29. A method according to claim 21 wherein the ratio of formononetin to said isoflavones is from 10:1 to 2:1.
30. A method according to claim 21 wherein said composition is in the form of a solid dosage form.
- 15 31. A method according to claim 21 wherein said dosage form comprises a tablet, capsule, granular preparation, buccal delivery vehicle or suppository.
32. A method according to claim 21 wherein said formononetin and biochanin
20 comprise an extract of chickpea or clover.
33. A method according to claim 32 wherein said clover is selected from red clover (*T. pratense*), subterranean clover (*T. subterranean*) or white clover (*T. repens*).
- 25 34. A method according to claim 33 wherein said clover is red clover.
35. A method according to claim 21 wherein the human subject is a pre-menopausal, menopausal or post-menopausal woman.

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36. A method according to claim 35 wherein said woman is normocholesterolemic or hypercholesterolemic.

37. A method according to claim 36 wherein said woman is artherosclerotic.

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38. A method according to claim 21 wherein said human subject is a normocholesterolemic or hypercholesterolemic male.

39. A method according to claim 38 wherein said male is hypercholesterolemic.

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40. A method according to claim 39 wherein said male is normocholesterolemic.

41. A method according to claim 21 which is a method for the prevention or treatment of bone fracture.

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42. A method according to claim 41 wherein said method is a method of preventing or treatment fractures involving bone with predominant cortical bone tissue.

43. A method according to claim 42 wherein said bone is selected from the femoral neck, femur, humerus, radius, ulna or tibia bone.

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(10)

(12) **PATENT ABSTRACT** (11) Document No. **AU-A-80655/87**
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(51) **4 International Patent Classification**

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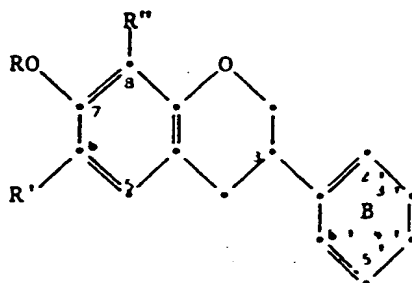
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(57) **Claim**

The compounds of this invention exhibit valuable pharmacological properties. They, for example, improve hemorheological parameters, inhibit platelet aggregation and phosphodiesterase, have calcium antagonist activity and improve peripheral oxygenation. These properties make the compounds useful e.g. for the treatment of vascular diseases as for example, intermittent claudication, arteriosclerosis, thrombotic diseases, myocardial ischemia, myocardial infarct, coronary diseases, cerebral ischemia, cerebral infarct, diabetic microangiopathy, arterial ulcer, Raynaud's syndrom, vasospasm, chronic venous insufficiency, venous ulcer or haemorrhoids.

Furthermore, the compounds of the invention are inhibitors of the 5-lipoxygenase and/or 12-lipoxygenase and have antioxydative activity. These properties make the compounds useful e.g. for the treatment of inflammatory diseases, hypersensitization and asthma as well as skin diseases, e.g. psoriasis.

1. A compound of the formula I



(I).

wherein the group OR represents hydroxy; lower alkoxy which is unsubstituted or substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, carboxy or lower alkoxycarbonyl; or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, diphenyl-lower alkyl, phenyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, phenylamino, lower alkanoylamino, benzoylamino; lower alkylsulfon-ylamino, phenylsulfon-ylamino; lower alkanoyl, benzoyl, carboxy, lower alkoxycarbonyl, carbamoyl, N-lower alkylcarbamoyl, N,N-di-lower alkylcarbamoyl, cyano, ureido, N-lower alkylureido, lower alkylsulfonyl; phenylsulfonyl; lower alkyl which is substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, halogen, carboxy or lower alkoxycarbonyl; lower alkoxy which is substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, halogen, carboxy or lower alkoxycarbonyl; C₃-C₇-alkoxy; and/or bivalent methylenedioxy; or wherein the ring B is monosubstituted by hydroxy, methoxy or ethoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy; or wherein the ring B is disubstituted by methoxy and lower alkoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy; with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; it being possible for all phenyl groups mentioned as such or in composed radicals to be unsubstituted or substituted by lower alkyl, lower alkoxy, halogen, hydroxy and/or nitro; or a salt thereof.

COMPLETE SPECIFICATION

(ORIGINAL)

Class

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Complete Specification for the invention entitled:

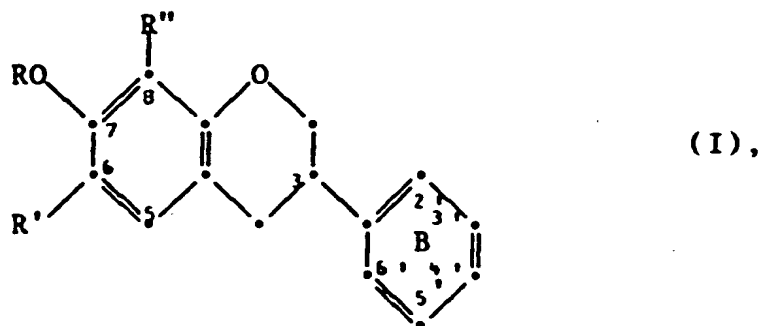
BICYCLIC COMPOUNDS

The following statement is a full description of this invention, including the best method of performing it known to :-

4-16152/+ /ZYM 43

Bicyclic Compounds

The present invention relates to isoflavans ($\hat{=}$ 3,4-dihydro-3-phenyl-2H-1-benzopyrans) of the formula I



wherein the group OR represents hydroxy; lower alkoxy which is unsubstituted or substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, carboxy or lower alkoxy-carbonyl; or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, diphenyl-lower alkyl, phenyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, phenylamin, lower alkanoylamino, benzylamino; lower alkylsulfon-ylamino, phenylsulfon-ylamino; lower alkanoyl, benzoyl, carboxy, lower alkoxycarbonyl, carbamoyl, N-lower alkylcarbamoyl, N,N-di-lower alkylcarbamoyl, cyan, ureido, N-lower alkylureido, lower

alkylsulfonyl; phenylsulfonyl; lower alkyl which is substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, halogen, carboxy or lower alkoxycarbonyl; lower alkoxy which is substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, halogen, carboxy or lower alkoxycarbonyl; C₃-C₇-alkoxy; and/or bivalent methylenedioxy;

or wherein the ring B is monosubstituted by hydroxy, methoxy or ethoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy;

or wherein the ring B is disubstituted by methoxy and lower alkoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy;

with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy;

it being possible for all phenyl groups mentioned as such or in composed radicals (like benzoyl, phenylamino etc.) to be unsubstituted or substituted by lower alkyl, lower alkoxy, halogen, hydroxy and/or nitro;

and salts thereof, processes for the manufacture of these compounds, pharmaceutical compositions comprising said compounds, and their use for the manufacture of pharmaceutical preparations or as pharmacologically active compounds.

The general definitions used herein have the following meanings within the scope of the present invention.

The term "lower" means that groups so defined have preferably up to and including 7, especially up to and including 4, carbon atoms.

Lower alkyl as such or in composed radicals like lower alkoxy etc. is e.g. n-propyl, isopropyl, n-butyl, isobutyl, sec.-butyl, tert.-butyl, n-pentyl, isopentyl, neopentyl, n-hexyl, isohexyl or n-heptyl, preferably ethyl and specially methyl.

Lower alkyl substituted by halogen is preferably trifluoromethyl.

Lower alkanoyl as such or in composed radicals like lower alkanoyl-xy etc. is e.g. formyl, acetyl, propionyl, n-butyryl, pivaloyl or valeroyl.

Halogen is preferably fluoro or chloro, but may be also bromo or iodo.

Phenylsulfonylamino means the radical $\text{-NHSO}_2\text{C}_6\text{H}_5$, lower alkylsulfonyl is -SO_2 -lower alkyl.

Ureido and lower alkylureido represent the radicals -NH-CONH_2 and -NH-CONHAlk (3-alkylureido) or -NAlk-CONH_2 (1-alkylureido) respectively (Alk=lower alkyl).

In lower alkoxy radicals which are substituted by hydroxy, epoxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino or halogen, the substituents mentioned are normally separated from the xy group in lower alkoxy by at least two carbon atoms.

Salts are preferably pharmaceutically acceptable salts, especially metal or ammonium salts of said compounds of formula I having a free carboxy group, more particularly alkali or alkaline earth metal salts, e.g., the sodium, potassium, magnesium or calcium salt; or advantageously easily crystallizing ammonium salts derived from ammonia or organic amines, such as mono-, di- or tri-lower (alkyl, cycloalkyl or hydroxyalkyl)-amines, lower alkylenediamines or lower (hydroxyalkyl or aralkyl)-alkylammonium hydroxides, e.g., methylamine, diethylamine, triethylamine, dicyclohexylamine, triethanolamine, ethylenediamine, tris-(hydroxymethyl)aminomethane or benzyltrimethylammonium hydroxide. Said compounds of formula I having a basic group form acid addition salts of preferably the pharmaceutically acceptable inorganic or organic acids, such as of strong mineral acids, for example hydrohalic, e.g. hydrochloric or hydrobromic acid; sulfuric, phosphoric, nitric or perchloric acid; aliphatic or aromatic carboxylic or sulfonic acids, e.g. formic,

acetic, propionic, succinic, glycolic, lactic, malic, tartaric, gluconic, citric, maleic, fumaric, pyruvic, phenylacetic, benzoic, 4-aminobenzoic, anthranilic, 4-hydroxybenzoic, salicylic, 4-amino-salicylic, pamoic, nicotinic, methanesulfonic, ethanesulfonic, hydroxyethanesulfonic, benzenesulfonic, p-toluenesulfonic, naphthalenesulfonic, sulfanilic or cyclohexylsulfamic acid, or other acidic organic substances, such as ascorbic acid.

For the purposes of isolation or purification it is also possible to use pharmaceutically unacceptable salts. Only the pharmaceutically acceptable, non-toxic salts are used therapeutically, however, and these are therefore preferred.

The compounds of this invention exhibit valuable pharmacological properties. They, for example, improve hemorheological parameters, inhibit platelet aggregation and phosphodiesterase, have calcium antagonist activity and improve peripheral oxygenation. These properties make the compounds useful e.g. for the treatment of vascular diseases as for example, intermittent claudication, arteriosclerosis, thrombotic diseases, myocardial ischemia, myocardial infarct, coronary diseases, cerebral ischemia, cerebral infarct, diabetic microangiopathy, arterial ulcer, Raynaud's syndrom, vasospasm, chronic venous insufficiency, venous ulcer or haemorrhoids.

Furthermore, the compounds of the invention are inhibitors of the 5-lipoxygenase and/or 12-lipoxygenase and have antioxidative activity. These properties make the compounds useful e.g. for the treatment of inflammatory diseases, hypersensitization and asthma as well as skin diseases, e.g. psoriasis.

1) Hemorheological parameters

The improvement of hemorheology parameters can be e.g. demonstrated with in vitro pharmacological models of human blood. In a first model, the analysis of the flow behaviour of concentrated suspensions of washed erythrocytes is described and measured according to

TEITEL [Blood Cells, 3, 55-70 (1977)] and SCHMID and SCHOENBEIN [VASA, 4, 263-270 (1975)]. In a second model, the hemorheological improvement is analysed with the yield shear stress technique according to KIESEWETTER et al. (Biblthca. haemat. 47, 14-20 (1981)) and KIESEWETTER et al. [Biorheology, 19, 363-374 (1982)]. Results are obtained by calculating ED50 in μM (concentration of substance which improves hemorheologic alteration by 50 % due to Ca^{++} stress). The ED50 values are, in the case of the compounds of the invention, approximately 0.05 μM or above.

2) cAMP-phosphodiesterase inhibition activity

The inhibitory effect of the compounds of the invention on cAMP-phosphodiesterase activity can be e.g. obtained according to WELLS et al. [Biochim. Biophys. Acta 384, 430-432 (1975)] and BERETZ et al. [Biochem. Pharmacol. 35, 257-262 (1986)]. Phosphodiesterases are obtained from fresh human platelets or fresh human aorta. The effect of the compounds on cAMP-phosphodiesterase of fresh human tissue is studied by adding solutions of compounds to be tested from 1 to 100 μM . IC50 values (concentration which is necessary to inhibit 50 % of the reaction) can be determined in order to evaluate the activity of the compounds. They are approximately 1 μM or above in the case of the compounds of the invention.

3) Inhibition of platelet aggregation

The inhibition of platelet aggregation can be e.g. demonstrated with the pharmacological model of washed human platelet collected from a forearm vein. Washed platelet suspensions are prepared according to CAZENAVE et al. [Ann. Biol. Clin. 41, 167 (1983)] and BERETZ et al. [Biochem. Pharmacol. 35, 257-262 (1986)]. Results can be obtained by calculating IC50 (concentration of the compound which inhibits 50 % of platelet aggregation). The IC50 values are, in the case of the compounds of the invention, approximately 1 μM or above.

4) Calcium antagonist activity

The evaluation of the property to inhibit the contraction induced by calcium chloride or potassium chloride depolarized rat mesentery can be made e.g. using the method described by BROCKAERT and GODFRAIND [Eur. J. Pharmacol. 53, 281 (1979)]. The inhibitory effect of the compounds is expressed as the concentration necessary to inhibit 50 % of the initial contraction obtained with CaCl_2 .

5) Peripheral oxygenation

The effect of the compounds on peripheral oxygenation can be measured e.g. according to SUNDER-PLASSMANN [Angiology, 32, 686-698 (1981)] and HAUSS [in "Oxyg. Transp. tissue in Experimental Biology and Medicine", Silver Ed. 1978, 419-422]. A pO_2 mean can be calculated after oral administration and compared between control and treated series of rats. A comparison of pO_2 can be realised between pretreatment and posttreatment values for the intravenous administration.

6) Inhibition of the 5-lipoxygenase pathway

The inhibition of the 5-lipoxygenase can be e.g. demonstrated according to KUHL et al. [Prostaglandins 28, 783-804 (1984)]. Porcine peripheral blood leucocytes are used in order to test the effect of the compounds of the invention. These effects are studied by adding solutions of the compounds to be tested of 0.1 to 1000 μM . IC_{50} values (concentration of the compound which inhibits 50 % of the 5-lipoxygenation) can be calculated in order to evaluate and compare the activity of the compounds. The IC_{50} values are lying, for the compounds of the invention, approximately between 0.35 and 200 μM or above.

7) Inhibition of the 12-lipoxygenase pathway

The inhibitory effect of the compounds of the invention related to the 12-lipoxygenase activity can be e.g. demonstrated according to KUHL et al. [Prostaglandins 28, 783-804 (1984)].

Porcine peripheral blood leucocytes are used for compounds evaluation. Solutions of the compounds to be tested are added with a final concentration of 0.1 to 1000 μM . Results are expressed as IC₅₀ values (concentration of the compound which inhibits 50 % of the 12-lipoxygenation). For the compounds of the invention, IC₅₀ values are lying approximately between 1 and 200 μM or above.

8) Antioxydative activity

The antioxydative activity can be e.g. demonstrated using the active oxygen method (AOM) according to WHEELER, Oil and Soap 9, 89 (1932). Stripped lard is used for estimation of the oxydative capacity of the compounds. A temperature stimuli (60°C for three days) is applied before titration with $\text{Na}_2\text{S}_2\text{O}_3$. The effect of the compounds on antioxydation activity is studied by adding a solution of the compound to be tested of 25 to 500 ppm (equivalent of 25 μg of compound/2 g lard to 500 μg of compound/2 g lard). Results are expressed as IC₅₀ values (concentration of the compound inhibiting 50 % of the oxydation reaction). For the compounds of the invention, IC₅₀ values are lying approximately in the range of 0.06 to 2.30 μM or above.

Preferred are the isoflavans of the formula I, wherein the group OR represents hydroxy, lower alkoxy or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, phenylamino, lower alkanoylamin, benzylamin; lower alkylsulfonylamino, phenylsulfonylamino; lower alkanoyl, benzoyl, carboxy, lower alkoxycarbonyl, carbamoyl, N-lower alkylcarbamoyl, N,N-di-lower alkylcarbamoyl, cyano, lower alkylsulfonyl; phenylsulfonyl; lower alkyl which is

substituted by halogen, carboxy or lower alkoxy carbonyl; lower alkoxy which is substituted by carboxy or lower alkoxy carbonyl; or bivalent methylenedioxy;

or wherein the ring B is monosubstituted by hydroxy or methoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy;

or wherein the ring B is disubstituted by methoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy;

with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; and pharmaceutically acceptable salts thereof.

Particularly preferred are the isoflavans of the formula I, wherein the group OR represents hydroxy, lower alkoxy or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, lower alkanoyloxy, halogen, amino, lower alkanoylamino, phenylsulfonylamino; carboxy, lower alkoxy carbonyl, carbamoyl, lower alkylsulfonyl; lower alkyl which is substituted by halogen or carboxy; lower alkoxy which is substituted by carboxy or lower alkoxy carbonyl; or bivalent methylenedioxy;

or wherein the ring B is monosubstituted by hydroxy or methoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy;

or wherein the ring B is disubstituted by methoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy;

with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; and pharmaceutically acceptable salts thereof.

Especially preferred are the isoflavans of the formula I, wherein the group OR represents hydroxy; lower alkoxy or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, or lower alkoxy which is substituted by carboxy or lower alkoxycarbonyl; or ring B is 3,4-dimethoxy-substituted;

with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; and pharmaceutically acceptable salts thereof.

Subgroups of the compounds of the invention are represented by

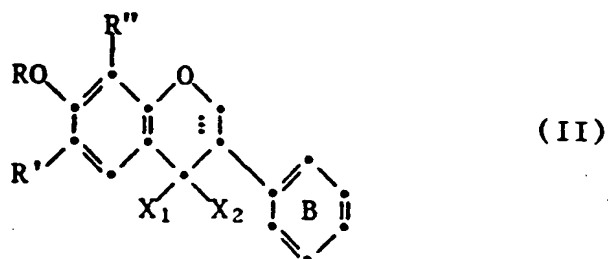
- (a) the compounds of the formula I which are 7,8-disubstituted, i.e. wherein R' represents hydrogen;
- (b) the compounds of the formula I which are 6,7-disubstituted, i.e. wherein R'' represents hydrogen; and
- (c) the compounds of the formula I wherein the ring B is substituted as defined above with the exclusion of all hydroxy, lower alkoxy and methylenedioxy substituents (but is not unsubstituted).

In particular preferred are the compounds of formula I, wherein the ring B is unsubstituted, monosubstituted in 3- or 4-position by one of the substituents mentioned or disubstituted in 3- and 4-position by methoxy. Especially preferred are the compounds of the formula I, wherein the ring B is monosubstituted in 4-position.

Above all are preferred the compounds of formula I described in the examples and pharmaceutically acceptable salts thereof.

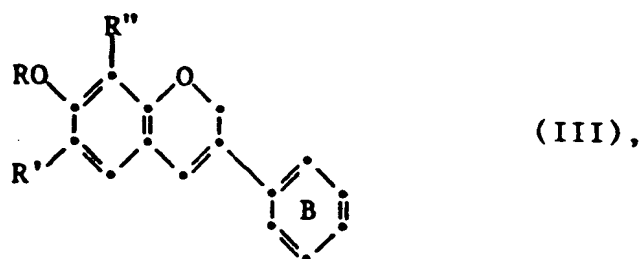
The compounds of the formula I can be produced by processes known per se, e.g.

a) by reducing a compound of the formula II



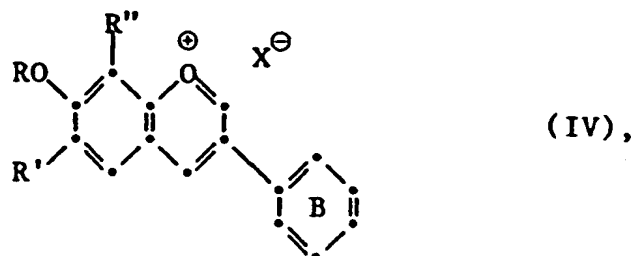
wherein the group CX_1X_2 represents a carbonyl group and the dotted line represents a bond or no bond, or wherein CX_1X_2 represents a hydroxymethylene group and the dotted line represents no bond, and OR, R', R" and ring B are as defined under formula I or represent radicals which are convertible to the groups OR, R', R" and/or ring B as defined under formula I by reduction, optionally with simultaneous reduction occurring within the groups OR, R', R" and/or ring B, or

b) by reducing a compound of the formula III



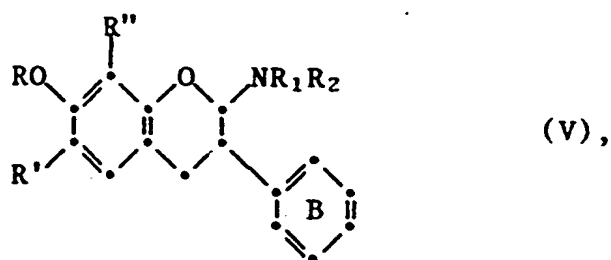
wherein OR, R', R" and ring B are as defined under formula I or represent radicals which are convertible to the groups OR, R', R" and/or ring B as defined under formula I by reduction, optionally with simultaneous reduction occurring within the groups OR, R', R" and/or ring B, or

c) by reducing a compound of the formula IV



wherein OR, R', R'' and ring B are as defined under formula I or represent radicals which are convertible to the groups OR, R', R'' and/or ring B as defined under formula I by reduction, and X^{\ominus} is an anion, optionally with simultaneous reduction occurring within the groups OR, R', R'' and/or ring B, or

d) by reducing a compound of the formula V

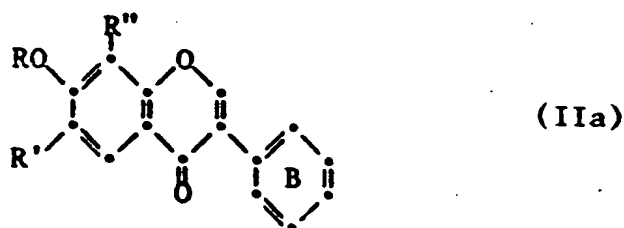


wherein the group NR_1R_2 represents a tertiary amino group and OR, R', R'' and ring B are as defined under formula I or represent radicals which are convertible to the groups OR, R', R'' and/or ring B as defined under formula I by reduction, optionally with simultaneous reduction occurring within the groups OR, R', R'' and/or ring B;

and/or, if desired, converting a resulting compound of formula I into another compound of formula I, and/or converting a resulting salt into the free compound or into another salt, and/or converting a resulting free compound of the formula I having salt-forming properties into a salt, and/or separating a resulting mixture of stereoisomers or optical isomers, such as a diastereoisomeric mixture, into the individual stereoisomers, optical isomers or enantiomeric mixtures, respectively, and/or splitting enantiomeric mixtures, such as a racemate, into the optical isomers.

Process a): The reduction can be accomplished e.g. with hydrogen in the presence of a hydrogenation catalyst, preferably Pd/C, and also e.g. platinum or platinum dioxide, optionally in the presence of a promoter, e.g. an acid, such as an inorganic acid, e.g. H_2SO_4 , HCl or $HClO_4$, an organic carboxylic acid, e.g. acetic acid or trifluoroacetic acid, or an organic sulfonic acid, e.g. phenylsulfonic acid, p-toluenesulfonic acid or methanesulfonic acid, optionally in the presence of an inert aprotic or protic solvent, or mixtures thereof, and at a hydrogen pressure of 1 to 50 bar [cp. Szabo et al., Acta Chim. Acad. Sci. Hung. 90, 381 (1976); Bull. Chem. Soc. Japan 37, 601, 606 (1964); Aust. J. Chem. 31, 455 (1978); Tetrahedron Lett. 1973, 1659].

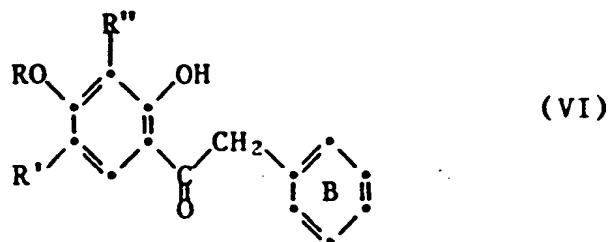
Another possibility to reduce the compounds of formula II wherein CX_1X_2 represents carbonyl and the dotted line is a bond, the isoflavone intermediates of formula IIa,



wherein OR, R' , R'' and ring B are as defined under formula I, is given by the Clemmensen reduction, i.e. with amalgamated zinc, concentrated HCl and optionally e.g. acetic acid [cp. Bull. Chem. Soc. Japan 37, 601, 606 (1964)].

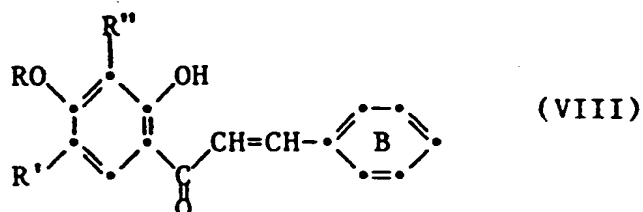
The isoflavones of formula IIa can be produced by processes known *per se*, e.g. by

1) reacting a compound of formula VI



wherein OR, R', R'' and ring B are as defined under formula I, with a derivative of formic acid under cyclization conditions, or

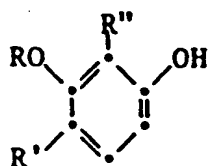
2) reacting a compound of formula VIII



wherein OR, R', R'' and ring B are as defined under formula I, with an agent suitable for forming the isoflavone by oxidative rearrangement and cyclization.

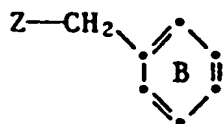
Process 1: The reaction consists of the condensation of the activated CH₂ group of the compound of formula VI with a derivative of formic acid followed by cyclization. Useful formic acid derivatives are e.g. dimethylformamide, triethyl orthoformate or ethyl formate. Cyclization agents that can be used are e.g. methanesulfonyl chloride/boron trifluoride etherate (cp. J.C.S. Chem. Comm. 1976, 78), POCl₃ [cp. C.A. 81, 135890h (1974) and C.A. 81, 25496b (1974)], pyridine and piperidine [cp. Indian J. Chem. 15B, 238 (1977); C.A. 87, 22970q (1977)], HClO₄ [cp. J. Chem. Res. (S) 1978, 47] or sodium [cp. Bull. Chem. Soc. Jap. 53, 831 (1980); Indian J. Chem. 6, 485 (1968)].

The intermediates of formula VI can be prepared e.g. by reacting a phenol of formula XII



(XII),

wherein OR, R' and R'' are as defined under formula I, with a phenylacetic acid derivative of the formula XIII

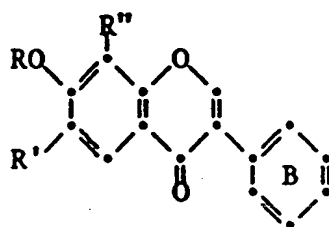


(XIII),

wherein Z preferably is cyano or halocarbonyl, especially -COCl, and the ring B is as defined under formula I, in the presence of a Lewis acid catalyst, e.g. a metal halide, such as ZnCl₂, AlCl₃ or FeCl₃, in a Houben-Hoesch or Friedel-Crafts acylation reaction, respectively [cp. Merck Index, 10th Edition, ONR 46 and 33].

Another possibility to obtain the compounds of formula VI is e.g. the Fries rearrangement [cp. Merck Index, 10th Edition, ONR 33] which comprises the reaction of a compound of formula XII as defined above with a compound of formula XIII, wherein Z represents halocarbonyl, in the absence of any Lewis acid catalyst resulting in the corresponding phenolic ester which rearranges under treatment with one of the Lewis acid catalysts mentioned above to the ortho-phenolic ketones of formula VI.

The novel isoflavones of the formula IIa



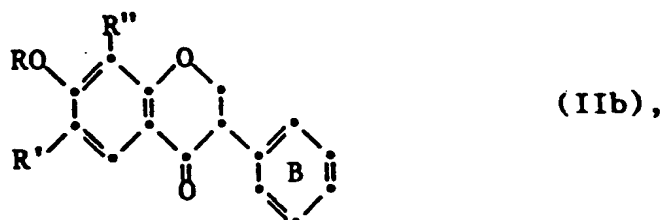
(IIa)

wherein OR, R', R'' and ring B are as defined und r formula I, are valuable intermediates for the preparation of the therapeutically active isoflavans of the formula I and thus form another embodiment of the present invention.

Especially preferred as intermediates are the novel isoflavones of the formula IIa described in the examples.

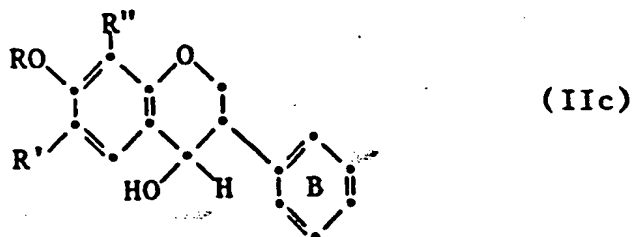
Process 2: The cyclization is achieved either in the presence of thallium(III) salts, e.g. Tl(NO₃)₃ or Tl(acetate)₃ [cp. J. Chem. Soc. Perkin Tr. 1 1974, 305; J. Chem. Soc. (C) 1970, 125 or Gazz. Chim. Ital. 112, 289 (1982)] or e.g. with the aid of hydrazoic acid [cp. Ann. Ist. Super. Sanita (1973), 9, Pt. 2-3, 174-175]. The starting materials of formula VIII can be prepared e.g. by condensation of the corresponding ortho-hydroxy-acetophenone with an optionally substituted benzaldehyde e.g. in the presence of a base, such as NaOH or KOH.

The compounds of the formula II, wherein CX₁X₂ represents carbonyl and the dotted line is no bond, the isoflavanone intermediates of formula IIb,



can be prepared e.g. by selectively reducing the double bond in an isoflavone intermediate of the formula IIa, e.g. with hydrogen and a Pd/C catalyst in the presence of a tertiary amine, e.g. triethylamine; or with H₂/Pd/C in dioxane, or with H₂/Pd/C in an aqueous ethanolic buffer pH 9-10 [cp. Szabo et al., Acta Chim. Acad. Sci. Hung. 90, 381 (1976)].

The compounds of formula II, wherein CX_1X_2 represents hydroxymethylene and the dotted line is no bond, the isoflavanol intermediates of formula IIc,



can be prepared e.g. by selectively reducing an isoflavone of formula IIa or an isoflavanone of formula IIb, e.g. with hydrogen and Raney nickel; or with $H_2/Pd/C$ in ethanol, or with $H_2/Pd/C$ in an aqueous ethanolic buffer pH 3-9 [cp. Szabo et al., loc. cit.].

Process b): The reduction according to process b) is accomplished e.g. by applying the same reduction means as listed above for process a), preferably by the use of $H_2/Pd/C$. The starting materials of formula III can be prepared e.g. by selective reduction of the carbonyl group in a corresponding coumarine, e.g. with KBH_4 [cp. Tetrahedron Lett. 24, 3993 (1983)]. Another possibility is given by reacting a compound of formula IIc first with an acetylating agent, e.g. acetic acid, and then eliminating acetic acid [cp. Bull. Chem. Soc. Jap. 37, 606 (1964)].

Process c): The anion X^\ominus can be e.g. the anion of any strong inorganic or organic acid, e.g. halide, such as chloride. The reduction according to process c) can be performed e.g. by applying the same reduction means as listed above for process a), preferably by the use of $H_2/Pt/Pd/C$. The starting isoflavylum salts of formula IV can be obtained e.g. by reaction of a compound of formula XII with an optionally substituted 2-phenylmalondialdehyde under acidic conditions, e.g. in the presence of HCl [cp. Austr. J. Chem. 34, 2647 (1981)].

Process d): The group NR_1R_2 represents preferably N,N-di-1st alkylamino, N-piperidino, N-piperazino and especially N-morpholino. The reduction according to process d) is accomplished e.g. by applying the same reduction means as listed above for process a), preferably $\text{H}_2/\text{Pd/C}$. The starting materials of formula V can be prepared e.g. by reacting a corresponding salicylaldehyde with an optionally substituted 2-tert. aminostyrene under heating [cp. J. Chem. Soc. Perkin Trans. 1 1982, 1193].

If any intermediates mentioned contain interfering reactive groups, e.g. carboxy, hydroxy, amino or mercapto groups, such may advantageously be temporarily protected at any stage with easily removable protecting groups. The choice of protecting groups for a particular reaction depends on several factors, e.g. the nature of the functional group to be protected, the structure and stability of the molecule of which the substituent is the functional group, and the reaction conditions. Protecting groups that meet these conditions and their introduction and removal are known to the art and are described, for example, in J.F.W. McOmie, "Protective Groups in Organic Chemistry", Plenum Press, London, New York 1973.

Depending upon the reaction conditions, the compounds of formula I are either obtained in the free form, or as a salt thereof. Any resulting base can be converted into a corresponding acid addition salt, preferably with the use of a therapeutically useful acid or an anion exchange preparation, or resulting salts can be converted into the corresponding free bases, for example, with the use of a stronger base, such as a metal or ammonium hydroxide or a basic salt, e.g. an alkali metal hydroxide or carbonate, or a cation exchange preparation. On the other hand, compounds of formula I containing acidic groups, e.g. carboxy or a phenolic hydroxy group, can be converted into salts in a manner known per se by treating with a base, e.g. an alkali metal hydroxide or alkoxide, an alkali metal or alkaline-earth metal salt, e.g. sodium hydrogen carbonate, ammonia or a suitable organic amine. The free compounds can be obtained by treating such salts with an acid. In view of the close

relationship between the free compounds and the compounds in the form of their salts, whenever a compound is referred to in this context, a corresponding salt is also intended, provided such is possible or appropriate under the circumstances.

The compounds of formula I contain at least one asymmetric carbon atom in position 3 and can be found as R- or S-enantiomers as well as enantiomeric mixtures thereof, such as a racemate. The present invention is intended to include all these forms, also those further isomers, and mixtures of at least two isomers, for example a diastereoisomeric mixture or enantiomeric mixture, which become possible if one or more further asymmetric center(s) are present within the molecule.

Any resulting mixtures of diastereoisomers, mixtures of racemates or geometric isomers can be separated on the basis of the physico-chemical differences of the constituents, in known manner, into single diastereoisomers, racemates, or geometric isomers, for example by chromatography and/or fractional crystallisation.

Any resulting enantiomeric mixtures, such as racemates, can be resolved into the optical isomers (antipodes) by known methods, for example by recrystallisation from an optically active solvent, or with the aid of microorganisms, or by e.g. reacting an acidic end product with an optically active base that forms salts with the racemic acid, and separating the salts obtained in this manner, for example by fractional crystallization, into the diastereoisomeric salts from which the optically active carboxylic acid antipodes can be liberated on acidification.

The above-mentioned reactions are carried out according to standard methods, in the presence or absence of diluents, preferably such as are inert to the reagents and are solvents thereof, of catalysts, condensing or said other reagents respectively and/or inert atmospheres, at low temperatures, room temperature or elevated temperatures, e.g. in a temperature range from -20° to $+200^{\circ}\text{C}$, preferably

between room temperature and the boiling point of the solvents used, and at atmospheric or super-atmospheric pressure. The preferred solvents, catalysts and reaction conditions are set forth in the appended illustrative examples.

The compounds, including their salts, can also be obtained in the form of their hydrates, or include other solvents used for their crystallization.

The invention further includes any variant of the present processes, in which an intermediate product obtainable at any stage thereof is used as starting material and the remaining steps are carried out, or the process is discontinued at any stage thereof, or in which a starting material is formed under the reaction conditions, or in which a reaction component is used in the form of a salt or an optically pure antipode. Mainly those starting materials should be used in said reactions, that lead to the formation of those compounds indicated above as being especially useful. The invention also relates to novel starting materials and processes for their manufacture.

The pharmacologically acceptable compounds of the present invention can be used e.g. for the manufacture of pharmaceutical preparations that contain an effective amount of the active ingredient alone or together with inorganic or organic, solid or liquid, pharmaceutically acceptable carriers. The pharmaceutical preparations are e.g. for enteral, such as oral or rectal, topical, transdermal and parenteral, such as intraperitoneal, intramuscular or intravenous, administration to warm-blooded animals including humans.

For oral administration there are used e.g. tablets or gelatine capsules that contain the active ingredient together with diluents, e.g. lactose, dextrose, sucrose, mannitol, sorbitol and/or cellulose, and lubricants, e.g. silica, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol; tablets also contain binders, e.g. starches, such as maize,

wheat, rice or arrowroot starch, gelatine, tragacanth, methyl-cellulose, sodium carboxymethylcellulose and/or polyvinylpyrrolidone, and, if desired, disintegrators, e.g. starches, agar, alginic acid or a salt thereof, such as sodium alginate, and/or e.g. effervescent mixtures, adsorbents, colourings, flavourings or sweeteners.

For parenteral administration there are suitable especially infusion solutions, preferably isotonic aqueous solutions or suspensions, it being possible to prepare these before use, e.g. from lyophilised preparations that contain the active ingredient alone or together with a carrier, e.g. mannitol. Such preparations may be sterilised and/or contain adjuncts, for example preservatives, stabilisers, wetting agents and/or emulsifiers, solubilisers, salts for regulating the osmotic pressure and/or buffers.

For topical and transdermal administration preferably hydrogels, emulsions, such as creams and ointments, and microemulsions such as isotropic transparent emulsion gels are used. Such preparations contain the active ingredient together with e.g. preservatives, stabilizers, thickening agents, emulsifiers, oils, solubilizers and penetration enhancers.

The present pharmaceutical preparations, which, if desired, may contain other pharmacologically active substances, are manufactured in a manner known per se, e.g. by means of conventional mixing, granulating, tableting, film coating, dissolving, confectioning or lyophilising processes, and contain from approximately 0.1 to 100 %, especially from approximately 1 to approximately 50 % or, in the case of lyophilisates, up to 100 %, of the active ingredient.

Depending upon the type of disorder, the individual condition of the organism and the mode of administration, the daily dose to be administered for the treatment of a warm-blooded animal (human or animal) weighing approximately 70 kg is from approximately 0.05 g to approximately 4 g.

The following Examples a) to f) are intended to illustrate the manufacture of some typical forms of administration, but do not in any way represent the only embodiments of those forms of administration.

a) 250 g of active substance are mixed with 550 g of lactose, 100 g of microcrystalline cellulose and 100 g of maize starch, and the mixture is moistened with an aqueous paste of 100 g maize starch, and granulated by being passed through a sieve. After drying, 60 g of talc, 10 g of magnesium stearate and 20 g of colloidal silica are added and the mixture is pressed to form 10,000 tablets each weighing 119 mg and each containing 25 mg of active substance, which may, if desired, be provided with dividing notches for a finer adjustment of the dosage.

b) A granulate is prepared from 100 g of active substance, 600 g of lactose, 300 g of cellulose, 200 g of maize starch and an aqueous paste of 120 g of maize starch. After drying, it is mixed with 30 g of colloidal silica, 90 g of talc and 15 g of magnesium stearate and processed so as to form 10,000 film coating cores. These are subsequently coated with an aqueous suspension of 20 g low substituted hydroxypropylmethylcellulose, 15 g of talc and 10 g of titanium dioxide and dried. The resulting film coated tablets each weigh 150 mg and contain 10 mg of active substance.

c) A sterile solution of 5.0 g of the active substance in 5000 ml of distilled water is introduced into 5 ml ampoules, the ampoules containing 5 mg of active ingredient in 5 ml of solution.

d) 25 g of active substance and 1975 g of finely ground suppository base (for example, cocoa butter) are thoroughly mixed and then melted. 1000 suppositories of 2 g are cast from the melt which has been kept homogenous by stirring. They each contain 25 mg of active substance.

e) 25 g of active substance and 120 g of granular lactose, e.g. Tabletose, 95 g of microcrystallin cellulose, e.g. Avicel® PH-102, 7 g of colloidal silicagel and 3 g of magnesium stearate are intimately mixed. The resulting powder is then sieved and filled in 250 mg portions into 1,000 gelatine capsules.

f) 400 g of active substance are dispersed in 24 l of distilled water with the addition of 70 g of a preservative, e.g. methylparaben, and 530 g of a thickening agent, e.g. carbomer 940, and the corresponding amount of 1N sodium hydroxide solution. 6000 g of petrolatum are mixed with 6000 g of a fatty alcohol, e.g. stearyl alcohol, with the addition of 3000 g of an emulsifier, e.g. polyoxyethylene sorbitan monolaurate. Both oil and water phase are heated separately to 70°C and then mixed together. After homogenisation and cooling, 1000 tubes are filled with 40 g of O/W ointment each.

The following examples are intended to illustrate the invention and are not to be construed as being limitations thereon. Temperatures are given in degrees Centigrade. In examples 1-7, the preparation of o-hydroxyphenyl-benzyl-ketone starting materials of the formula VI is described. Examples 8-17 show the preparation of isoflavone intermediates of the formula IIa. In examples 18-35, the preparation of isoflavans of the formula I is described. Examples 36-71 show the preparation of isoflavans of the formula I including the respective starting materials and intermediates.

Example 1: 1,2,4-Trihydroxybenzene (40.3 g) is suspended in dry diethylether (250 ml) containing dry ZnCl_2 (38.2 g) and 4-methylphenylacetonitrile (49.8 g). The suspension is then exposed for 6 h at 0° to a gentle stream of dry HCl, the gas bubbling through the suspension under continuous stirring. Then the reaction mixture is kept for 50 h at 4° and then after the supernatant is decanted from heavy oil which is separated. The oil is washed twice with diethylether, then with water (1 l), and conc. HCl (20 ml) are added and the mixture is boiled for 1 h under reflux. After cooling to room temperature, the mixture is extracted with diethylether

(3 x 250 ml). The combined ther solutions are extracted with NaOH 2N solutions (3 x 100 ml). The alkali extract is acidified with conc. HCl and the mixture co l ed on ice. The crude product is filtered off and recrystallized from methanol / water. The product is dried in vacuo to constant weight to yield 2,4,5-trihydroxy-phenyl-4'-methylbenzylketone, m.p. 173°.

Example 2: As example 1, but using pyrogallol (39.1 g) instead of 1,2,4-trihydroxybenzene, 52.5 g of 4-methylphenylacetonitrile and 41.0 g of ZnCl₂. The suspension is exposed for 10 h at 0° to a stream of HCl. 2,3,4-Trihydroxyphenyl-4'-methylbenzylketone is obtained, m.p. 148°.

Example 3: As example 1, but using 2,6-dihydroxytoluene (24.8 g) instead of 1,2,4-trihydroxybenzene, 27.5 g of methylphenylacetonitrile and AlCl₃ (26.7 g) instead of ZnCl₂. The suspension is exposed for 16 h at 0° to a stream of HCl. After cooling the reaction mixture to room temperature, it is extracted with chloroform instead of diethylether. After evaporating the chloroform extract to a small volume, the crystals of the product are obtained. After drying 2,4-dihydroxy-3-methylphenyl-4'-methylbenzylketone is obtained; m.p. 154°.

Example 4: As example 1, but using 2,6-dihydroxytoluene (49.6 g) instead of 1,2,4-trihydroxybenzene, 3,4-dimethoxyphenylacetonitrile (70.9 g) instead of 4-methylphenylacetonitrile and 47.7 g of ZnCl₂. The suspension is exposed for 6 h at 0° to a stream of HCl. 2,4-Dihydroxy-3-methylphenyl-3',4'-dimethoxybenzylketone is obtained; m.p. 168-170°.

Example 5: As example 1, but using 2,6-dihydroxytoluene (44.7 g) instead of 1,2,4-trihydroxybenzene, 4-chlorophenylacetonitrile (54.6 g) instead of 4-methylphenylacetonitrile and 47.7 g of ZnCl₂. The suspension is exposed for 12 h at 0° to a stream of HCl. 2,4-Dihydroxy-3-methylphenyl-4'-chlor benzylketone is obtained; m.p. 167°.

Example 6: As example 1, but using 45.4 g of 1,2,4-trihydroxybenzene, 4-chlorophenylacetonitrile (56.1 g) instead of 4-methylphenylacetonitrile and AlCl_3 (44.7 g) instead of ZnCl_2 . The suspension is exposed for 12 h at 0° to a stream of HCl . The crude substance is purified by column chromatography (silica gel; chloroform). 2,4,5-Trihydroxyphenyl-4'-chlorobenzylketone is obtained; m.p. 170° .

Example 7: As example 1, but using 2,6-dihydroxytoluene (14.9 g) instead of 1,2,4-trihydroxybenzene, 4-nitrophenylacetonitrile (19.5 g) instead of 4-methylphenylacetonitrile and AlCl_3 (13.3 g) instead of ZnCl_2 . The suspension is exposed for 12 h at 0° to a stream of HCl . 2,4-Dihydroxy-3-methylphenyl-4'-nitrobenzylketone is obtained; m.p. $190-192^\circ$.

Example 8: 2,4,5-Trihydroxyphenyl-4'-methylbenzylketone (3.9 g) is dissolved in dry dimethylformamide (50 ml). To this solution is added dropwise borontrifluoride-diethyletherate (7.4 g); the reaction is exothermic. After adjusting the temperature to 50° , a solution of methanesulfonylchloride (5.2 g) in dry dimethylformamide (25 ml) is added dropwise. This mixture is heated at $90 - 100^\circ$ for 2 h, cooled to room temperature and poured into water (500 ml) while stirring. The separated product is filtered off, washed with water and recrystallized from methanol. The product is dried in vacuo to constant weight to yield 6,7-dihydroxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one; m.p. 280° .

Example 9: As example 8, but using 2,3,4-trihydroxyphenyl-4'-methylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one is obtained; m.p. 225° .

Example 10: As example 8, but using 2,4-dihydroxy-3-methylphenyl-4'-methylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(4-methylphenyl)-4H-1-benzopyran-4-one is obtained; m.p. 264-267°.

Example 11: As example 8, but using 2,4-dihydroxy-3-methylphenyl-4'-methoxybenzylketone [J. Indian Chem. Soc. 39, 301 (1962)] instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one is obtained; m.p. 235°.

Example 12: As example 8, but using 2,4-dihydroxy-3-methylphenyl-3',4'-dimethoxybenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one is obtained; m.p. 234°.

Example 13: As example 8, but using 2,4-dihydroxy-3-methylphenyl-4'-chlorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(4-chlorophenyl)-4H-1-benzopyran-4-one is obtained; m.p. 275°.

Example 14: As example 8, but using 2,4,5-trihydroxyphenyl-4'-chlorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(4-chlorophenyl)-4H-1-benzopyran-4-one is obtained; m.p. 299°.

Example 15: As example 8, but using 2,4-dihydroxy-3-methylphenyl-4'-nitrobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(4-nitrophenyl)-4H-1-benzopyran-4-one is obtained; m.p. 345°.

Example 16: 7-Hydroxy-8-methyl-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one [see example 12] (3.12 g) is dissolved in dimethylformamide (15 ml) and K₂CO₃ (3.5 g) is added to the solution. While stirring, epibromohydrine (2.47 g) is dropped into the mixture. After heating for 5 h at 60°, the content of the reaction flask is poured into water (500 ml) and the precipitated product is filtered

off and recrystallized from ethanol. The product is dried in vacuo to constant weight to yield 7-(2,3-epoxypropoxy)-8-methyl-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one, m.p. 214°.

Example 17: 6,7-Methylenedioxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one [cp. Nippon Kagaku Zasshi 85, 793 (1964), Agr. Biol. Chem. (Tokyo) 32, 740 (1968) and Angew. Chem. 93, 129 (1981)] (2.0 g) is dissolved in dimethylformamide (15 ml) and K₂CO₃ (2.8 g) is added to the solution. While stirring, 2-bromoethylbutyrate (2.7 g) is dropped into the mixture. The content of the reaction flask is refluxed for 30 min. After cooling, water (50 ml) is added and the mixture is extracted with chloroform (3 x 30 ml), the extract washed with water (3 x 15 ml) and dried over Na₂SO₄. Chloroform is removed under reduced pressure and the residue is recrystallized from methanol. The product is dried in vacuo to constant weight to yield 6,7-methylenedioxy-3-[4-(1-ethoxycarbonyl-1-propyloxy)-phenyl]-4H-1-benzopyran-4-one, m.p. 119-121°.

Example 18: 6,7-Dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one [Indian J. Chem. Sect. B 19B, 82 (1980) and Indian J. Chem. Sect. B 15B, 1049 (1977)] (25 g) in a mixture of dioxane and ethanol 1:1 (1250 ml) is hydrogenated for 8 days at normal pressure and room temperature over palladium 10 % on active charcoal (2.5 g) in the presence of concentrated H₂SO₄ (2.5 ml). After filtration of the catalyst, the filtrate is evaporated under reduced pressure to a volume of about 50 ml and diluted with water till turbidity appears. After cooling the solution, a solid precipitates. It is filtered off and recrystallized from ethanol. The product is dried in vacuo to constant weight to yield 3,4-dihydro-6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran, m.p. 153°.

Example 19: As example 18, with the same proportions of chemicals, but using 6,7-dihydroxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one (16 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzo-

pyran-4-one. The substance is hydrogenated for 24 h. 3,4-Dihydro-6,7-dihydroxy-3-(4-methylphenyl)-2H-1-benzopyran is obtained; m.p. 166°.

Example 20: As example 18, with the same proportions of chemicals, but using 6,7-methylenedioxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one [Nippon Kagaku Zasshi 85, 793 (1964)] (17 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 2 days. 3,4-Dihydro-6,7-methylenedioxy-3-(4-methoxyphenyl)-2H-1-benzopyran is obtained; m.p. 131°.

Example 21: As example 18, with the same proportions of chemicals, but using 7,8-dihydroxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one [J. Sci. Ind. Research (India) 20B, 334 (1961)] (5 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 7 days. 3,4-Dihydro-7,8-dihydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran is obtained; m.p. 156°.

Example 22: As example 18, with the same proportions of chemicals, but using 7,8-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one [Tetrahedron 18, 1443 (1962)] (10 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 7 days. 3,4-Dihydro-7,8-dihydroxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran is obtained; m.p. 176°.

Example 23: As example 18, with the same proportions of chemicals, but using 7,8-dihydroxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one (1.9 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 23 h. 3,4-Dihydro-7,8-dihydroxy-3-(4-methylphenyl)-2H-1-benzopyran is obtained; m.p. 164°.

Example 24: As example 18, with the same proportions of chemicals, but using 7-hydroxy-8-methyl-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one (10 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 23 h. 3,4-Dihydro-7-hydroxy-8-methyl-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran is obtained; m.p. 164°.

nyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 10 days. 3,4-Dihydro-7-hydroxy-8-methyl-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran is obtained; m.p. 125-127°.

Example 25: As example 18, with the same proportions of chemicals, but using 7-hydroxy-8-methyl-3-(4-chlorophenyl)-4H-1-benzopyran-4-one (5 g) instead of 6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-one. The substance is hydrogenated for 5 days. 3,4-Dihydro-7-hydroxy-8-methyl-3-(4-chlorophenyl)-2H-1-benzopyran is obtained; m.p. 159°.

Example 26: 7-Hydroxy-8-methyl-3-(4-methylphenyl)-4H-1-benzopyran-4-one (400 mg) in a mixture of dioxane and ethanol 1:1 (200 ml) is hydrogenated for 64 h at room temperature over palladium 10 % on active charcoal (100 mg) in the presence of p-toluenesulfonic acid monohydrate (120 mg). After filtration of the catalyst, the filtrate is evaporated under reduced pressure to a volume of about 50 ml. Water (150 ml) is added and the solution is extracted with dichloromethane (3 x 100 ml). The combined organic solutions are washed with water, dried and evaporated. The product is dried in vacuo to constant weight to yield 3,4-dihydro-7-hydroxy-8-methyl-3-(4-methylphenyl)-2H-1-benzopyran, m.p. 136-137°.

Example 27: As example 26, but using 7,8-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one [J. Sci. Ind. Research (India) 20B, 334 (1961)] (540 mg) instead of 7-hydroxy-8-methyl-3-(4-methylphenyl)-4H-1-benzopyran-4-one. A crystalline precipitate is formed when water is added; it is filtrated off and washed with water. 3,4-Dihydro-7,8-dihydroxy-3-(4-hydroxyphenyl)-2H-1-benzopyran is obtained; m.p. 225-226°.

Example 28: As example 26, but using 7-hydroxy-8-methyl-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one (28 mg) instead of 7-hydroxy-8-methyl-3-(4-methylphenyl)-4H-1-benzopyran-4-one. A crystalline

precipitate is formed when water is added; it is filtrated off and washed with water. 3,4-Dihydro-7-hydroxy-8-methyl-3-(4-methoxyphenyl)-2H-1-benzopyran is obtained; m.p. 140-141°.

Example 29: As example 26, but using 6,7-methylenedioxy-3-[4-(1-ethoxycarbonyl-1-propyloxy)-phenyl]-4H-1-benzopyran-4-one (25 mg) instead of 7-hydroxy-8-methyl-3-(4-methylphenyl)-4H-1-benzopyran-4-one. After filtration of the catalyst, the solvent is evaporated and the residue is purified by column chromatography on silica gel using dichloromethane as eluant. 3,4-Dihydro-6,7-methylenedioxy-3-[4-(1-ethoxycarbonyl-1-propyloxy)-phenyl]-2H-1-benzopyran is obtained as an oil; ¹H-NMR (d₆-DMSO): δ = 6.9, 7.0, 7.4 (d), 7.6 (d) [6H, Aromaten-H]; 3,4 (m, 2H, CH₂); 2.5 (m, 2H, CH₂); 1.8 (t, 3H, CH₃); 1.6 (t, 3H, CH₃). MS:m/e = 384 (M⁺).

Example 30: 7-Hydroxy-8-methyl-3-(4-nitrophenyl)-4H-1-benzopyran-4-one (440 mg), dissolved in a mixture of dioxane/ethanol 1:1 (200 ml), is hydrogenated for 15 h at room temperature over Raney nickel (200 mg). After filtration of the catalyst, the filtrate is evaporated under reduced pressure. The residue is dissolved in ethanol (75 ml) and hydrogenated for 15 h at room temperature over palladium 10 % on active charcoal (200 mg) in the presence of p-toluenesulfonic acid monohydrate (760 mg). After filtration of the catalyst, the filtrate is evaporated under reduced pressure. The residue is dissolved in water (100 ml). The solution is neutralized to pH 7.5 by addition of a saturated solution of NaHCO₃ and extracted with ethyl acetate (3 x 50 ml). The combined organic solutions are washed with water (2 x 20 ml), dried over MgSO₄ and evaporated. The residue is purified by column chromatography on silica gel using dichloromethane / ethyl acetate 1:1 as eluent. The product is dried in vacuo to constant weight to yield 3,4-dihydro-7-hydroxy-8-methyl-3-(4-aminophenyl)-2H-1-benzopyran, m.p. 150-151°.

Example 31: 3,4-Dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran [cp. US patent 4,264,509] (10.0 g) is dissolved in dry pyridine (30 ml) and acetic anhydride (30 ml) is added. The solution

is kept at room temperature for 48 h. Pyridine and the excess of the reagent are removed by evaporating with ethanol under reduced pressure. The residue is purified by column chromatography on silica gel using chloroform as eluent. The crude product is recrystallized from methanol. The product is dried in vacuo to constant weight to yield 3,4-dihydro-6,7-diacetoxy-3-(4-methoxyphenyl)-2H-1-benzopyran, m.p. 104°.

Example 32: As example 31, but using 3,4-dihydro-6,7-dihydroxy-3-(4-hydroxyphenyl)-2H-1-benzopyran [cp. Phytochemistry 23, 2203 (1984) and US patent 4,264,509] (10.3 g) instead of 3,4-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran. 3,4-Dihydro-6,7-diacetoxy-3-(4-acetoxyphenyl)-2H-1-benzopyran is obtained; m.p. 145-147°.

Example 33: As example 31, but using 3,4-dihydro-6,7-dihydroxy-3-phenyl-2H-1-benzopyran [cp. US patent 4,264,509] (9.7 g) instead of 3,4-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran. 3,4-Dihydro-6,7-diacetoxy-3-phenyl-2H-1-benzopyran is obtained; m.p. 102°.

Example 34: Triethylamine (4.0 g) and dichlorodiphenylmethane (3.5 g) are added to a solution of 3,4-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran [cp. US patent 4,264,509] (1.4 g) in pyridine (50 ml) containing 1 % of water. The solution is warmed at 50° for 6 h and then poured into a mixture of water and 5N HCl 2:1 (150 ml). It is extracted with dichloromethane (3 x 50 ml) and the combined organic solutions are washed with water. The solvent is evaporated to dryness under reduced pressure. The residue is recrystallized from ethanol. After drying in vacuo to constant weight, 3,4-dihydro-6,7-diphenylmethylenedioxy-3-(4-methoxyphenyl)-2H-1-benzopyran is obtained; m.p. 138-139°.

Example 35: Triethylamine (2.02 g) and 3,4-dihydro-7,8-dihydroxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran [see example 22] (0.75 g) are dissolved in pyridine (20 ml) containing 1 % of water. A solution of

dichlorodiphenylmethane (1.77 g) in pyridin (5 ml) is slowly added. The mixture is heated at 70° for 4 h. The solution is then poured into water (250 ml) and evaporated in vacuo to a volume of about 100 ml. It is then extracted with ethyl acetate (3 x 100 ml), the combined organic solutions are washed with water (2 x 50 ml) and evaporated to dryness under reduced pressure. The residue is purified by column chromatography on silica gel using dichloromethane/hexane 1:1 as eluent. The product is recrystallized from hexane. After drying in vacuo to constant weight, 3,4-dihydro-7,8-diphenylmethylenedioxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran is obtained; m.p. 137-138°.

Example 36: 7,8-Dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one (100 mg), dissolved in a mixture of ethanol/dioxan 2:1 (75 ml), is hydrogenated for 24 h at room temperature over palladium 10 % on active charcoal (50 mg) in the presence of p-toluenesulphonic acid monohydrate (50 mg). After filtration of the catalyst, the filtrate is evaporated under vacuum to a minimum volume, then diluted with water (75 ml) and neutralized to pH 7 by addition of a saturated solution of NaHCO₃. An oil is obtained which is extracted with ethyl acetate. The extract is dried over magnesium sulphate and evaporated under vacuum to yield an amorphous solid which is dissolved in methylene chloride and eluted on a silica gel column. After evaporation of the main fraction, one obtains 3,4-dihydro-7,8-dihydroxy-3-(3-methylphenyl)-2H-1-benzopyran, m.p. 95-96°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,3,4-trihydroxyphenyl-3'-methylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 268°.

(b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 3-methylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-3'-methylbenzylketone is obtained, m.p. 141°.

Example 37: As example 36, but using 7,8-dihydroxy-3-(3-carboxyphenyl)-4H-1-benzopyran-4-one (100 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After neutralization to pH 7 by addition of a saturated solution of NaHCO_3 , there is a precipitation of crystals of 3,4-dihydro-7,8-dihydroxy-3-(3-carboxyphenyl)-2H-1-benzopyran, m.p. 194-195°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,3,4-trihydroxyphenyl-3'-carboxybenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(3-carboxyphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 313-314°.

(b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 3-carboxyphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-3'-carboxybenzylketone is obtained, m.p. 222°.

Example 38: As example 36, but using 7,8-dihydroxy-3-(4-nitrophenyl)-4H-1-benzopyran-4-one [cp. J. Sci. Ind. Research (India) 20B, 334 (1961)] (300 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After neutralization to pH 7 by addition of a saturated solution of NaHCO_3 , a precipitate is obtained which is filtered, washed with water and dried under vacuum over phosphorous pentoxide. The mother liquor is acidified to pH 4.5 and extracted with ethyl acetate. The organic layer is evaporated to dryness under vacuum to yield a solid. The two fractions consist of 3,4-dihydro-7,8-dihydroxy-3-(4-aminophenyl)-2H-1-benzopyran, m.p. 210-212°.

Example 39: 6,7-Dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one (1.2 g), dissolved in a mixture of dioxan/ethanol 4:6 (40 ml), is hydrogenated for 24 h at room temperature over palladium 5 % on active charcoal (0.48 g) in the presence of a few drops of concentrated sulphuric acid. After filtration of the catalyst, the filtrate is evaporated to dryness under vacuum. An oil is obtained which is dissolved in methylene chloride. The solution is washed with water and dried over magnesium sulphate to give an oil which on crystallization yields 3,4-dihydro-6,7-dihydroxy-3-(3-methylphenyl)-2H-1-benzopyran, m.p. 146-148°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-methylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 258°.

(b) As example 1, but using 3-methylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-3'-methylbenzylketone is obtained, m.p. 197°.

Example 40: 6,7-Dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one (1.2 g), dissolved in a mixture of dioxan/ethanol 4:6 (40 ml), is hydrogenated for 24 h at room temperature over palladium 5 % on active charcoal (0.48 g) in the presence of a few drops of conc. sulphuric acid. After filtration of the catalyst, the filtrate is evaporated to dryness under vacuum. An oil is obtained which crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(4-fluorophenyl)-2H-1-benzopyran, m.p. 135-137°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4,5-trihydroxyphenyl-4'-fluorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 296°.

(b) As example 1, but using 4-fluorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-4'-fluorobenzylketone is obtained, m.p. 189-190°.

Example 41: As example 40, but using 7,8-dihydroxy-3-phenyl-4H-1-benzopyran-4-one [cp. Zh. Org. Khim. 40, 2459 (1970) and Zh. Org. Khim. 5, 515 (1969)] (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the residue is washed with water and dried under vacuum to give 3,4-dihydro-7,8-dihydroxy-3-phenyl-2H-1-benzopyran, m.p. 138°.

Example 42: As example 36, but using 6,7-dihydroxy-3-(3,4-methylenedioxyphenyl)-4H-1-benzopyran-4-one [cp. Bull. Chem. Soc. Japan 38, 612 (1965)] (900 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After neutralization to pH 7, a precipitate is obtained which is purified by elution on a silica gel column (CHCl₃/diisopropylether 1:1) to yield 3,4-dihydro-6,7-dihydroxy-3-(3,4-methylenedioxyphenyl)-2H-1-benzopyran, m.p. 163-164°.

Example 43: As example 40, but using 6-n-hexyl-7-hydroxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the residue is eluted on a silicagel column (CH₂Cl₂): the main fraction yields an oil which crystallizes and represents 3,4-dihydro-6-n-hexyl-7-hydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran, m.p. 64-66°.

The starting materials are prepared as follows:

(a) As example 8, but using 5-n-hexyl-2,4-dihydroxyphenyl-4'-methoxybenzyl ketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6-n-Hexyl-7-hydroxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 188°.

(b) As example 1, but using 4-n-hexylresorcinol instead of 1,2,4-trihydroxybenzene and 4-methoxyphenylacetonitrile instead of 4-methylphenylacetonitrile. 5-n-Hexyl-2,4-dihydroxyphenyl-4'-methoxybenzylketone is obtained, m.p. 137°.

Example 44: As example 36, but using 7,8-dihydroxy-3-(3-fluorophenyl)-4H-1-benzopyran-4-one (544 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After extraction with ethyl acetate, the organic solution is washed with water and evaporated under vacuum to yield 3,4-dihydro-7,8-dihydroxy-3-(3-fluorophenyl)-2H-1-benzopyran, m.p. 148-149°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,3,4-trihydroxyphenyl-3'-fluorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(3-fluorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 230°.

(b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 3-fluorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-trihydroxyphenyl-3'-fluorobenzylketone is obtained, m.p. 162.

Example 45: As example 40, but using 6,7-dihydroxy-3-(3-fluorophenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the oily residue is dissolved in methylene chloride. The solution is washed with water, dried over magnesium sulphate and vaporated to dryness under vacuum. The residue is purified by elution on a silica gel column (CHCl₃/diisopropyl ether 1:1) and the

oil representing the main fraction on crystallization yields 3,4-dihydro-6,7-dihydroxy-3-(3-fluorophenyl)-2H-1-benzopyran, m.p. 121-123°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-fluorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(3-fluorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 277°.

(b) As example 1, but using 3-fluorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-3'-fluorobenzylketone is obtained, m.p. 209°.

Example 46: As example 36, but using 7-hydroxy-8-methyl-3-(3,4-methylenedioxyphenyl)-4H-1-benzopyran-4-one (100 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After neutralization to pH 7, a precipitate is formed which is filtered, washed with water and dried under vacuum to give 3,4-dihydro-7-hydroxy-8-methyl-3-(3,4-methylenedioxyphenyl)-2H-1-benzopyran, m.p. 130-131°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4-dihydroxy-3-methylphenyl-3',4'-methylenedioxybenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(3,4-methylenedioxyphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 270-272°.

(b) As example 1, but using 2,6-dihydroxytoluene instead of 1,2,4-trihydroxybenzene and 3,4-methylenedioxyphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4-Dihydroxy-3-methylphenyl-3',4'-methylenedioxybenzylketone is obtained, m.p. 164°.

Example 47: As example 36, but using 7-hydroxy-8-methyl-3-(3-ethoxycarbonylphenyl)-4H-1-benzopyran-4-one (100 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After neutralization, a crystalline precipitate is formed which is filtered, washed with water and dried under vacuum to give 3,4-dihydro-7-hydroxy-8-methyl-3-(3-ethoxycarbonylphenyl)-2H-1-benzopyran, m.p. 143-144°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4-dihydroxy-3-methylphenyl-3'-ethoxycarbonylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(3-ethoxycarbonylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 222°.

(b) As example 1, but using 2,6-dihydroxytoluene instead of 1,2,4-trihydroxybenzene and 3-ethoxycarbonylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4-Dihydroxy-3-methylphenyl-3'-ethoxycarbonylbenzylketone is obtained, m.p. 157°.

Example 48: As example 36, but using 7-hydroxy-8-methyl-3-(3-trifluoromethylphenyl)-4H-1-benzopyran-4-one (100 mg) instead of 7,8-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. After extraction with ethyl acetate, the organic layer is washed with water, dried over magnesium sulphate, then evaporated under vacuum to give 3,4-dihydro-7-hydroxy-8-methyl-3-(3-trifluoromethylphenyl)-2H-1-benzopyran as an oil. $^1\text{H-NMR}$ (d_6 -DMSO): δ = 7.6 (m, 4H, Aromaten-H); 6.7 (d, 1H, Arom.-H); 6.4 (d, 1H, Arom.-H); 2.0 (s, 3H, CH_3). MS: m/e = 308 (M^+).

The starting materials are prepared as follows:

(a) As example 8, but using 2,4-dihydroxy-3-methylphenyl-3'-trifluoromethylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(3-trifluoromethylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 283-284°.

(b) As example 1, but using 2,6-dihydroxytoluene instead of 1,2,4-trihydroxybenzene and 3-trifluoromethylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4-Dihydroxy-3-methylphenyl-3'-trifluoromethylbenzylketone is obtained, m.p. 167°.

Example 49: As example 40, but using 7,8-dimethoxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the residue is washed with water and dried under vacuum to yield 3,4-dihydro-7,8-dimethoxy-3-(4-methylphenyl)-2H-1-benzopyran, m.p. 98-99°.

The starting material is prepared as follows:

a) 7,8-Dihydroxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one (2.7 g), described in example 9, is dissolved in dry acetone (50 ml). Pyrolyzed potassium carbonate (4.14 g) is added and the mixture is heated to reflux. A solution of methyl sulphate (3.78 g) in acetone (10 ml) is then added dropwise and heating is maintained for three more hours. After cooling, the solid is filtered and the solution is evaporated to dryness under vacuum. Water is added to the residue which is filtered, washed with an aqueous sodium hydroxide solution (1 N) then with water and dried. After crystallization in methanol, 7,8-dimethoxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 135°.

Example 50: As example 39, but using 7-hydroxy-8-methyl-3-(4-chlorophenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. Tetrahydrofuran and acetic acid are used instead of dioxan/ethanol and sulphuric acid. The resulting oil is finally purified by elution on a silica gel column (CHCl₃) to yield 3,4-dihydro-7-hydroxy-8-methyl-3-(4-chlorophenyl)-2H-1-benzopyran, m.p. 98-100°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4-dihydroxy-3-methylphenyl-4'-chloro-benzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7-Hydroxy-8-methyl-3-(4-chlorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 296-297°.

(b) As example 1, but using 2,6-dihydroxytoluene instead of 1,2,4-trihydroxybenzene and 4-chlorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4-Dihydroxy-3-methylphenyl-4'-chloro-benzylketone is obtained, m.p. 167°.

Example 51: As example 39, but using 6,7-dihydroxy-3-(4-amino-phenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil corresponds to 3,4-dihydro-6,7-dihydroxy-3-(4-aminophenyl)-2H-1-benzopyran. It is converted to the corresponding hydrochloride and also to the corresponding hemisulfate.

The starting material is prepared as follows:

a) 6,7-Dihydroxy-3-(4-nitrophenyl)-4H-1-benzopyran-4-one [cp. J. Inst. Chem. (Calcutta) 43, 234 (1971)] (1 g) is dissolved in 250 ml of a mixture of dioxan and ethanol 1:1. Raney nickel (0.45 g) is added and the mixture is hydrogenated for 18 h at room temperature, then filtered. The solution is evaporated under vacuum to about one third of its original volume until a precipitate occurs which is filtered and crystallized in dioxan to give 6,7-dihydroxy-3-(4-aminophenyl)-4H-1-benzopyran-4-one, m.p. 305-306°.

Example 52: As example 39, but using 6,7-dihydroxy-3-(3-chloro-phenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. Tetrahydrofuran and acetic acid are used instead of dioxan/ethanol and sulphuric acid. The resulting oil crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(3-chlorophenyl)-2H-1-benzopyran.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-chlorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(3-chlorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 308°.

(b) As example 1, but using 3-chlorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-3'-chlorobenzylketone is obtained, m.p. 212°.

Example 53: As example 40, but using 6,7-dihydroxy-3-(3-trifluoromethylphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the residue is dissolved in methylene chloride. The solution is washed with water, dried over magnesium sulphate and evaporated under vacuum to yield 3,4-dihydro-6,7-dihydroxy-3-(3-trifluoromethylphenyl)-2H-1-benzopyran, m.p. 139-141°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-trifluoromethylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(3-trifluoromethylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 274-275°.

(b) As example 1, but using 3-trifluoromethylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-3'-trifluoromethylbenzylketone is obtained, m.p. 170°.

Example 54: As example 39, but using 7,8-dihydroxy-3-(3-trifluoromethylphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil is finally purified by elution on a silica gel column (CH₂Cl₂) to give 3,4-dihydro-7,8-dihydroxy-3-(3-trifluoromethylphenyl)-2H-1-benzopyran, m.p. 99-100°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,3,4-trihydroxyphenyl-3'-trifluoromethylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(3-trifluoromethylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 225°.

(b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 3-trifluoromethylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-3'-trifluoromethylbenzylketone is obtained, m.p. 171°.

Example 55: As example 39, but using 7,8-dihydroxy-3-(3-chlorophenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. Tetrahydrofuran and acetic acid are used instead of dioxan/ethanol and sulphuric acid. The resulting oil crystallizes to give 3,4-dihydro-7,8-dihydroxy-3-(3-chlorophenyl)-2H-1-benzopyran, m.p. 99-101°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,3,4-trihydroxyphenyl-3'-chlorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(3-chlorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 258°.

(b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 3-chlorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-3'-chlorobenzylketone is obtained, m.p. 155°.

Example 56: As example 40, but using 7,8-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the residue is washed with water, dried under vacuum and

then purified by elution on a silica gel column (CH_2Cl_2) to give 3,4-dihydro-7,8-dihydroxy-3-(4-fluorophenyl)-2H-1-benzopyran, m.p. 166-168°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,3,4-trihydroxyphenyl-4'-fluorobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one is obtained, m.p. 289°.

(b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 4-fluorophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-4'-fluorobenzylketone is obtained, m.p. 152°.

Example 57: As example 39, but using 6,7-methylenedioxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one [see Example 17] (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil crystallizes to give 3,4-dihydro-6,7-methylenedioxy-3-(4-hydroxyphenyl)-2H-1-benzopyran, m.p. 139-140°.

Example 58: As example 39, but using 6,7-dihydroxy-3-(4-isopropylphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(4-isopropylphenyl)-2H-1-benzopyran, m.p. 129 - 131°.

The starting materials are prepared as follows:

a) As example 8, but using 2,4,5-trihydroxyphenyl-4'-isopropylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(4-isopropylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 181-182°.

b) As example 1, but using 4-isopropylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-4'-isopropylbenzylketone is obtained, m.p. 131°.

Example 59: As example 39, but using 6,7-dihydroxy-3-(4-carboxymethylphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(4-ethoxycarbonylmethylphenyl)-2H-1-benzopyran, m.p. 164 - 166°.

The starting materials are prepared as follows:

a) 6,7-Dihydroxy-3-(4-cyanomethylphenyl)-4H-1-benzopyran-4-one (1 g) is dissolved in a mixture of conc. hydrochloric acid and acetic acid 1:1 (10 ml), then the solution is boiled for 2 h. After evaporation of the solution under vacuum and crystallization of the residue in a mixture of ethanol and water, 6,7-dihydroxy-3-(4-carboxymethylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 278-280°.

b) As example 8, but using 2,4,5-trihydroxyphenyl-4'-cyanomethylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(4-cyanomethylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 258-260°.

c) As example 1, but using 4-cyanomethylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-4'-cyanomethylbenzylketone is obtained, m.p. 195-196°.

Example 60: As example 39, but using 6,7-dihydroxy-3-(4-phenylsulfonylaminophenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(4-phenylsulfonylaminophenyl)-2H-1-benzopyran, m.p. 152-154°.

The starting material is prepared as follows:

a) 6,7-Dihydroxy-3-(4-aminophenyl)-4H-1-benzopyran-4-one [see Example 51a] (538 mg) is dissolved in a mixture of dimethylformamide and water 15:85 (5 ml) and the solution is heated to 90° in the presence of benzenesulfonyl chloride (210 mg). After 15 minutes the pH has dropped to 3 and it is adjusted to 9 by addition of a 25 % sodium hydroxide solution. Benzenesulfonyl chloride (additional 143 mg) and 25 % sodium hydroxide solution (total amount used: 1 ml) are added in the same way until the pH does not vary any more. Aqueous hydrochloric acid is then added to lower the pH to 3 and the solution is cooled until precipitation occurs. The precipitate is filtered, washed with water and dried to give 6,7-dihydroxy-3-(4-phenylsulfonylaminophenyl)-4H-1-benzopyran-4-one, m.p. 285-286°.

Example 61: As example 40, but using 6,7-dihydroxy-3-(3-ethoxycarbonylphenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(4-fluorophenyl)-4H-1-benzopyran-4-one. After evaporation of the filtrate, the residue crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(3-ethoxycarbonylphenyl)-2H-1-benzopyran, m.p. 131-133°.

The starting materials are prepared as follows:

(a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-ethoxycarbonylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. After purification by column chromatography (silica gel/toluene:methanol 100:5), 6,7-dihydroxy-3-(3-ethoxycarbonylphenyl)-4H-1-benzopyran-4-one is obtained, m.p. 217-218°.

(b) As example 1, but using 3-ethoxycarbonylphenylacetonitrile instead of 4-methylphenylacetonitrile. After purification by column chromatography (silica gel/methylene chloride:methanol 20:1) and crystallization from a mixture of methanol and water, 2,4,5-trihydroxyphenyl-3'-ethoxycarbonylbenzylketone is obtained, m.p. 177-178°.

Example 62: As example 39, but using 6,7-dihydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one (1.2 g) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil crystallizes to give 3,4-dihydroxy-6,7-dihydroxy-3-(4-acetylaminophenyl)-2H-1-benzopyran, m.p. 201-203° (88% purity).

The starting material is prepared as follows:

a) 7-Acetyloxy-6-hydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one [see Example 65a] (20 mg) is dissolved in a mixture of 1N aqueous hydrochloric acid and ethanol 1:1 (10 ml) and stirred during 12 h at room temperature. The solution is then extracted with dichloromethane, the dichloromethane layer is washed with water and evaporated to dryness under vacuum to give 6,7-dihydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one, m.p. >310° (dec.).

Example 63: As example 39, but using 6,7-dihydroxy-3-(3-methylsulfonylphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydroxy-6,7-dihydroxy-3-(3-methylsulfonylphenyl)-2H-1-benzopyran is obtained.

The starting materials are prepared as follows:

a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-methylsulfonylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(3-methylsulfonylphenyl)-4H-1-benzopyran-4-one is obtained.

b) As example 1, but using 3-methylsulfonylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-3'-methylsulfonylbenzylketone is obtained.

Example 64: As example 39, but using 6,7-dihydroxy-3-(3-tert-butylphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydroxy-6,7-dihydroxy-3-(3-tert-butylphenyl)-2H-1-benzopyran is obtained.

The starting materials are prepared as follows:

a) As example 8, but using 2,4,5-trihydroxyphenyl-3'-tert-butylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 6,7-Dihydroxy-3-(3-tert-butylphenyl)-4H-1-benzopyran-4-one is obtained.

b) As example 1, but using 3-tert-butylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,4,5-Trihydroxyphenyl-3'-tert-butylbenzylketone is obtained.

Example 65: As example 39, but using 7-acetyloxy-6-hydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one (20 mg) instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. The resulting oil crystallizes to give 3,4-dihydro-6,7-dihydroxy-3-(4-acetylaminophenyl)-2H-1-benzopyran, m.p. 201-203° (88% purity).

The starting material is prepared as follows:

a) 6,7-Dihydroxy-3-(4-aminophenyl)-4H-1-benzopyran-4-one [see Example 51a] (269 mg) is dissolved in ethanol (3 ml). Acetic anhydride (4 ml) is added and the solution heated to 70° for 2.5 h, then left overnight at room temperature. The resulting precipitate is filtered, washed with water and dried to give 7-acetyloxy-6-hydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one, m.p. 265 - 266°.

Example 66: As example 39, but using 7,8-dihydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydro-7,8-dihydroxy-3-(4-acetylaminophenyl)-2H-1-benzopyran is obtained.

The starting materials are prepared as follows:

a) As in example 8, but using 2,3,4-trihydroxyphenyl-4'-acetylaminobenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(4-acetylaminophenyl)-4H-1-benzopyran-4-one is obtained.

b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 4-acetylaminophenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-4'-acetylaminobenzylketone is obtained.

Example 67: As example 39, but using 7,8-dihydroxy-3-(4-carbamoylphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydro-7,8-dihydroxy-3-(4-carbamoylphenyl)-2H-1-benzopyran is obtained.

The starting materials are prepared as follows:

a) As in example 8, but using 2,3,4-trihydroxyphenyl-4'-carbamoylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(4-carbamoylphenyl)-4H-1-benzopyran-4-one is obtained.

b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 4-carbamoylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-4'-carbamoylbenzylketone is obtained.

Example 68: As example 39, but using 7,8-dihydroxy-3-(3-n-propylsulfonylphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydro-7,8-dihydroxy-3-(3-n-propylsulfonylphenyl)-2H-1-benzopyran is obtained.

The starting materials are prepared as follows:

a) As exampl 8, but using 2,3,4-trihydroxyphenyl-3'-n-propylsulfonylbenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Dihydroxy-3-(3-n-propylsulfonylphenyl)-4H-1-benzopyran-4-one is obtained.

b) As example 1, but using pyrogallol instead of 1,2,4-trihydroxybenzene and 3-n-propylsulfonylphenylacetonitrile instead of 4-methylphenylacetonitrile. 2,3,4-Trihydroxyphenyl-3'-n-propylsulfonylbenzylketone is obtained.

Example 69: As example 39, but using 7,8-dimethoxy-3-(3-carboxyphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydro-7,8-dimethoxy-3-(3-carboxyphenyl)-2H-1-benzopyran is obtained.

The starting material is prepared as follows:

a) As example 49a, but using 7,8-dihydroxy-3-(3-carboxyphenyl)-4H-1-benzopyran-4-one [see Example 37a] instead of 7,8-dihydroxy-3-(4-methylphenyl)-4H-1-benzopyran-4-one. 7,8-Dimethoxy-3-(3-carboxyphenyl)-4H-1-benzopyran-4-one is obtained.

Example 70: As example 39, but using 7,8-dimethoxy-3-(4-carboxymethoxyphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydro-7,8-dimethoxy-3-(4-carboxymethoxyphenyl)-2H-1-benzopyran is obtained.

The starting material is prepared as follows:

a) NaH is dissolved in dry dimethylformamide under nitrogen atmosphere at 0°. 7,8-Dimethoxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one [cp. J. Inst. Chem. (Calcutta) 43, 234 (1971)], dissolved in dry dimethylformamid, is added sl wly, then a solution of chloroacetic acid. Th solution is heat d at 70° for 5 h and evaporated under vacuum to about one third of its original volum. After

cooling, water is added and a precipitate occurs which is filtered and crystallized in methanol to give 7,8-dimethoxy-3-(4-carboxymethoxyphenyl)-4H-1-benzopyran-4-one.

Example 71: As example 39, but using 7,8-methylenedioxy-3-(4-carboxyphenyl)-4H-1-benzopyran-4-one instead of 6,7-dihydroxy-3-(3-methylphenyl)-4H-1-benzopyran-4-one. 3,4-Dihydro-7,8-methylenedioxy-3-(4-carboxyphenyl)-2H-1-benzopyran is obtained.

The starting materials are prepared as follows:

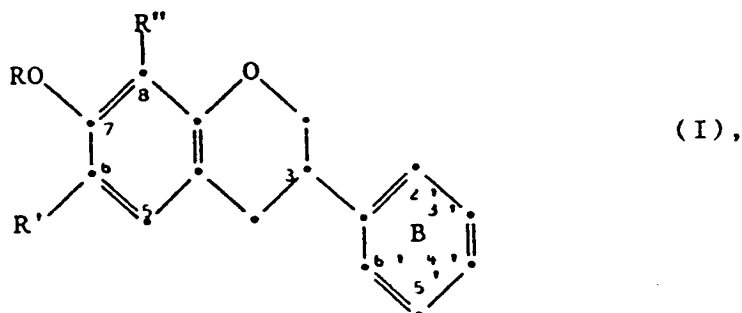
a) As example 8, but using 2-hydroxy-3,4-methylenedioxyphenyl-4'-carboxybenzylketone instead of 2,4,5-trihydroxyphenyl-4'-methylbenzylketone. 7,8-Methylenedioxy-3-(4-carboxyphenyl)-4H-1-benzopyran-4-one is obtained.

b) As example 1, but using 2-hydroxy-3,4-methylenedioxybenzene instead of 1,2,4-trihydroxybenzene and 4-carboxyphenylacetonitrile instead of 4-methylphenylacetonitrile. 2-Hydroxy-3,4-methylenedioxyphenyl-4'-carboxybenzylketone is obtained.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

~~Claims~~

1. A compound of the formula I



wherein the group OR represents hydroxy; lower alkoxy which is unsubstituted or substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, carboxy or lower alkoxycarbonyl; or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, diphenyl-lower alkyl, phenyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, phenylamino, lower alkanoylamino, benzoylamino; lower alkylsulfon-ylamino, phenylsulfon-ylamino; lower alkanoyl, benzoyl, carboxy, lower alkoxycarbonyl, carbamoyl, N-lower alkylcarbamoyl, N,N-di-lower alkylcarbamoyl, cyano, ureido, N-lower alkylureido, lower alkylsulfonyl; phenylsulfonyl; lower alkyl which is substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, halogen, carboxy or lower alkoxycarbonyl; lower alkoxy which is

substituted by hydroxy, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, halogen, carboxy or lower alkoxycarbonyl; C₃-C₇-alkoxy; and/or bivalent methylenedioxy;

or wherein the ring B is monosubstituted by hydroxy, methoxy or ethoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy; or wherein the ring B is disubstituted by methoxy and lower alkoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy; with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; it being possible for all phenyl groups mentioned as such or in composed radicals to be unsubstituted or substituted by lower alkyl, lower alkoxy, halogen, hydroxy and/or nitro; or a salt thereof.

2. A compound according to claim 1 of the formula I, wherein the group OR represents hydroxy, lower alkoxy or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or substituted by lower alkyl and/or phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, phenylamino, lower alkanoylamino, benzoylamino; lower alkylsulfonylamino, phenylsulfonylamino; lower alkanoyl, benzoyl, carboxy, lower alkoxycarbonyl, carbamoyl, N-lower alkylcarbamoyl, N,N-di-lower alkylcarbamoyl, cyano, lower alkylsulfonyl; phenylsulfonyl; lower alkyl which is substituted by halogen, carboxy or lower alkoxycarbonyl; lower alkoxy which is substituted by carboxy or lower alkoxy-carbonyl; or bivalent methylenedioxy;

or wherein the ring B is monosubstituted by hydroxy or methoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy;
or wherein the ring B is disubstituted by methoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy;
with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; or a pharmaceutically acceptable salt thereof.

3. A compound according to claim 1 of the formula I, wherein the group OR represents hydroxy, lower alkoxy or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, lower alkanoyloxy, halogen, amino, lower alkanoylamino, phenylsulfonylamino; carboxy, lower alkoxycarbonyl, carbamoyl, lower alkylsulfonyl; lower alkyl which is substituted by halogen or carboxy; lower alkoxy which is substituted by carboxy or lower alkoxycarbonyl; or bivalent methylenedioxy;

or wherein the ring B is monosubstituted by hydroxy or methoxy, provided that R' is other than hydroxy, methoxy or ethoxy, if the group OR represents hydroxy, methoxy or ethoxy;
or wherein the ring B is disubstituted by methoxy, provided in case of 2',4'-dimethoxy substitution that R' and R'' are other than methoxy, if the group OR represents methoxy;
with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy;
r a pharmaceutically acceptable salt thereof.

4. A compound according to claim 1 of the formula I, wherein the group OR represents hydroxy; lower alkoxy or lower alkanoyloxy; one of the radicals R' and R'' represents hydroxy, lower alkoxy, lower alkanoyloxy or lower alkyl and the other one is hydrogen; or the groups OR and R' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R'' is hydrogen; or the groups OR and R'' together form a bivalent methylenedioxy radical which is unsubstituted or disubstituted by phenyl, and R' is hydrogen; and the ring B is unsubstituted or substituted by lower alkyl, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, or lower alkoxy which is substituted by carboxy or lower alkoxycarbonyl; or ring B is 3,4-dimethoxy-substituted; with the proviso that the ring B must be substituted, if R' is hydroxy and the group OR represents hydroxy or methoxy; or a pharmaceutically acceptable salt thereof.
5. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran.
6. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran.
7. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-methylphenyl)-2H-1-benzopyran.
8. A compound according to claim 1 being 3,4-dihydro-6,7-diphenyl-methylenedioxy-3-(4-methoxyphenyl)-2H-1-benzopyran.
9. A compound according to claim 1 being 3,4-dihydro-6,7-diacetoxy-3-(4-methoxyphenyl)-2H-1-benzopyran.
10. A compound according to claim 1 being 3,4-dihydro-6,7-methylene-dioxy-3-(4-methoxyphenyl)-2H-1-benzopyran.
11. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran.

12. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(4-methylphenyl)-2H-1-benzopyran.

13. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran.

14. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(4-chlorophenyl)-2H-1-benzopyran.

15. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(4-methylphenyl)-2H-1-benzopyran.

16. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(4-hydroxyphenyl)-2H-1-benzopyran.

17. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(4-methoxyphenyl)-2H-1-benzopyran.

18. A compound according to claim 1 being 3,4-dihydro-6,7-methylene-dioxy-3-[4-(1-ethoxycarbonyl-1-propyloxy)-phenyl]-2H-1-benzopyran.

19. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(4-aminophenyl)-2H-1-benzopyran.

20. A compound according to claim 1 being 3,4-dihydro-6,7-diacetoxy-3-(4-acetoxyphenyl)-2H-1-benzopyran.

21. A compound according to claim 1 being 3,4-dihydro-6,7-di-acetoxy-3-phenyl-2H-1-benzopyran.

22. A compound according to claim 1 being 3,4-dihydro-7,8-diphenyl-methylenedioxy-3-(3,4-dimethoxyphenyl)-2H-1-benzopyran.

23. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(3-methylphenyl)-2H-1-benzopyran.

24. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(3-carboxyphenyl)-2H-1-benzopyran.
25. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(4-aminophenyl)-2H-1-benzopyran.
26. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3-methylphenyl)-2H-1-benzopyran.
27. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-fluorophenyl)-2H-1-benzopyran.
28. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-phenyl-2H-1-benzopyran.
29. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3,4-methylenedioxyphenyl)-2H-1-benzopyran.
30. A compound according to claim 1 being 3,4-dihydro-6-n-hexyl-7-hydroxy-3-(4-methoxyphenyl)-2H-1-benzopyran.
31. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(3-fluorophenyl)-2H-1-benzopyran.
32. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3-fluorophenyl)-2H-1-benzopyran.
33. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(3,4-methylenedioxyphenyl)-2H-1-benzopyran.
34. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(3-ethoxycarbonylphenyl)-2H-1-benzopyran.
35. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(3-trifluoromethylphenyl)-2H-1-benzopyran.

36. A compound according to claim 1 being 3,4-dihydro-7,8-dimethoxy-3-(4-methylphenyl)-2H-1-benzopyran.
37. A compound according to claim 1 being 3,4-dihydro-7-hydroxy-8-methyl-3-(4-chlorophenyl)-2H-1-benzopyran.
38. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-aminophenyl)-2H-1-benzopyran.
39. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3-chlorophenyl)-2H-1-benzopyran.
40. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3-trifluoromethylphenyl)-2H-1-benzopyran.
41. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(3-trifluoromethylphenyl)-2H-1-benzopyran.
42. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(3-chlorophenyl)-2H-1-benzopyran.
43. A compound according to claim 1 being 3,4-dihydro-7,8-dihydroxy-3-(4-fluorophenyl)-2H-1-benzopyran.
44. A compound according to claim 1 being 3,4-dihydro-6,7-methylene-dioxy-3-(4-hydroxyphenyl)-2H-1-benzopyran.
45. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-isopropylphenyl)-2H-1-benzopyran.
46. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-carboxymethylphenyl)-2H-1-benzopyran.
47. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-phenylsulfonylaminophenyl)-2H-1-benzopyran.

48. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(3-ethoxycarbonylphenyl)-2H-1-benzopyran.

49. A compound according to claim 1 being 3,4-dihydro-6,7-dihydroxy-3-(4-acetylamino-phenyl)-2H-1-benzopyran.

50. A pharmaceutical preparation containing a compound of formula I according to any one of claims 1 - 49, or a pharmaceutically acceptable salt thereof, together with one or more pharmaceutically acceptable carriers.

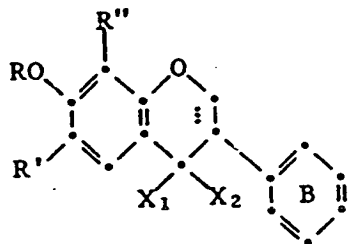
51. A compound of the formula I according to any one of claims 1 - 49 or a pharmaceutically acceptable salt thereof for use in a method for the prophylactic and/or therapeutic treatment of the animal or human body.

52. A compound of the formula I according to any one of claims 1 - 49 or a pharmaceutically acceptable salt thereof for use in the treatment of vascular diseases.

53. Use of a compound of the formula I according to any one of claims 1 - 49 or of a pharmaceutically acceptable salt thereof for the manufacture of a pharmaceutical preparation.

54. A process for the manufacture of a compound of the formula I according to any one of claims 1 - 49, or a salt thereof, which comprises

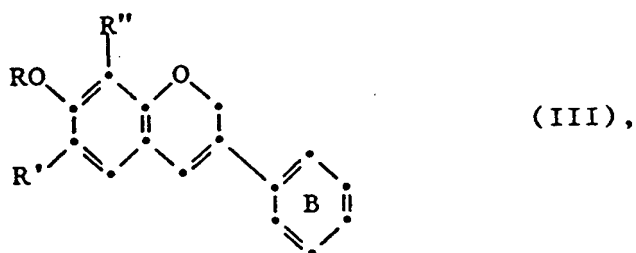
a) reducing a compound of the formula II



(II)

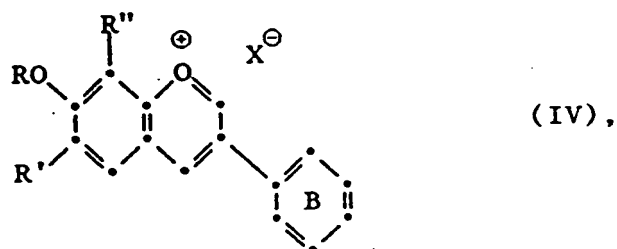
wherein the group CX_1X_2 represents a carbonyl group and the dotted line represents a bond or no bond, or wherein CX_1X_2 represents a hydroxymethylene group and the dotted line represents no bond, and OR , R' , R'' and ring B are as defined under formula I or represent radicals which are convertible to the groups OR , R' , R'' and/or ring B as defined under formula I by reduction, optionally with simultaneous reduction occurring within the groups OR , R' , R'' and/or ring B , or

b) reducing a compound of the formula III



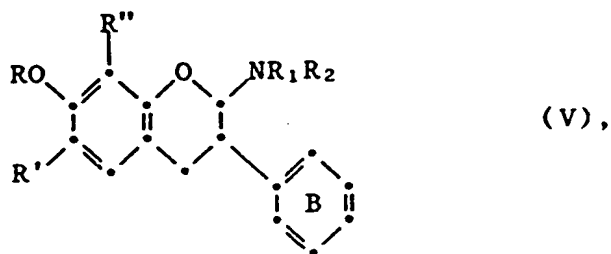
wherein OR , R' , R'' and ring B are as defined under formula I or represent radicals which are convertible to the groups OR , R' , R'' and/or ring B as defined under formula I by reduction, optionally with simultaneous reduction occurring within the groups OR , R' , R'' and/or ring B , or

c) reducing a compound of the formula IV



wherein OR , R' , R'' and ring B are as defined under formula I or represent radicals which are convertible to the groups OR , R' , R'' and/or ring B as defined under formula I by reduction, and X^\ominus is an anion, optionally with simultaneous reduction occurring within the groups OR , R' , R'' and/or ring B , or

d) reducing a compound of the formula V



wherein the group NR_1R_2 represents a tertiary amino group and OR, R' , R'' and ring B are as defined under formula I or represent radicals which are convertible to the groups OR, R' , R'' and/or ring B as defined under formula I by reduction, optionally with simultaneous reduction occurring within the groups OR, R' , R'' and/or ring B;

and/or, if desired, converting a resulting compound of formula I into another compound of formula I, and/or converting a resulting salt into the free compound or into another salt, and/or converting a resulting free compound of the formula I having salt-forming properties into a salt, and/or separating a resulting mixture of stereoisomers or optical isomers, such as a diastereoisomeric mixture, into the individual stereoisomers, optical isomers or enantiomeric mixtures, respectively, and/or splitting enantiomeric mixtures, such as a racemate, into the optical isomers.

55. A novel compound whenever prepared by a method according to claim 54.

56. A novel compound of formula I substantially as herein described.

57. A method for the preparation of a novel compound of the formula I substantially as herein described.

58. A compound prepared by the process claimed in claim 57.

59. A pharmaceutical preparation containing a compound of formula I substantially as herein described.

FO 7.4 BL/cc*/gm*/kg*/cs*

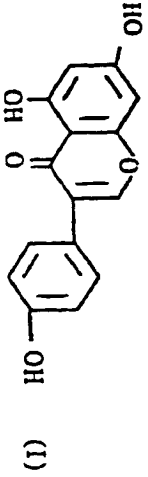
DATED this 2nd day of November 1987.

ZYMA SA

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95-272884/36	B02	KIKK 93.12.17 *JP 07173148-A	B(4-A10, 6-A1, 7-A2) .3
KIKKOMAN CORP	93.12.17 93JP-343304 (95.07.11) C07D 311/40, 311/36	Simple and convenient isolation of genistein from isoflavone mixt. - by selective extraction with chloro-hydrocarbon C95-123478	
Production of genistein (G) from isoflavone mixt. comprises extraction with a solvent of formula: $\text{CHnCl}(4-n)$ wherein $n = 0, 1$ or 2 .		As the source of isoflavone mixt., most pref. is high content soybean and it is treated previously by extraction with water, organic solvent or a mixt. thereof. Aq. waste from soybean processing can also be utilised in this purpose. The isoflavone mixt. is extracted with water, recovered by ion-exchange resin, freed from lipophilic impurities by extraction with organic solvent and pref. finally hydrolysed to aglycone mixt.. Process is carried out with 30-500 vol. (to the isoflavone mixt.) of the chlorohydrocarbon (chloroform, dichloromethane or carbon tetrachloride) pref. by repeated extraction under reflux for generally 3 hr. opt. followed by further purification to give (G).	
<u>USE/ADVANTAGE</u>		(G) is one of the active aglycone component of isoflavone glycosides such as genistin from Leguminosae, Compositae or Iridaceae, having various pharmacological activities such as oestrogenic, antibacterial, antioxidant and antineoplastic activities. The present process utilises newly discovered exceptional higher solubility of (G) in the chlorohydrocarbon solvent than other aglycones and attains simple and convenient isolation method of (G) solely consisting of extraction from the isoflavone mixt. contg. it, which has been prepared by time-requiring, non-economical and troublesome chromatography. For example, a 1:1-mixt. of (G) and daidzein (D) was extracted 3-times under reflux for 3 hrs. using various solvent. The selectivity (G/D-ratio in the extract) was shown along with the solvent used: chloroform (65.4), dichloromethane (25.18), carbon tetrachloride (9.80), methanol (1.02) and butanol (1.05). In an example, 2kg skin-stripped soybean was soaked at 50 ° C for 2hr. in 20 L water previously adjusted to pH=9 by addition of alkali, the liquor part was collected, stirred with addition of 500 g DAIA-	
		JP 07173148-A+	

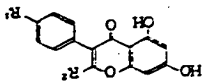
<p>93-252721/32 B03 KIKKOMAN CORP 91.12.20 91JP-354486 (93.07.09) C07D 311/40, 311/36 Prepn. for iso-flavone derivs. by extn. from soy oil or sauce - exhibits antioxidant, oestrogenic, anti-hyperlipidaemic, antibacterial, anti-haemolytic and anti-hypercholesterolaemic activity C93-112358</p>	<p>B(6-A1, 12-A1, 12-G7, 12-H3) e.g., phenolic acid, etc. with 20% ethanol, adsorbed components were eluted with 80% ethanol, and dried in vacuo by an evaporator. Fraction of isoflavonoglucon (9.5g) was obtd.. Purity was at least 90% (HPLC). (3pp Dwg.No.0/0)</p>
<p>Isoflavone derivs. are prepd. by extracting isoflavone cpds. from soy sauce or soy oil using organic solvent. USE/ADVANTAGE - Isoflavone aglycone as drug effect having useful cpds. can be obtd. from soy sauce of soy oil as by-prod. in soy prepn. by a simple operation, in good efficiency. Isoflavone cpds. e.g., daidzin, daidzein, genistein, genistein, have oestrogenic activity, antioxidant activity, antihemolytic activity, antibacterial activity, anti-hyperlipidaemic activity, anti-hypercholesterolaemic activity, also have an anticancer effect. In an example, a soy sauce as a by-prod. in soy prepn. by using a defatted soybean as a material (500g), was extracted with 80% methanol by refluxing 3 times. The extracted soln. was filtered by Toyo-filter paper No.2, then, conc. in vacuo by an evaporator to obtain purified material (40g). This was dissolved in a small amt. of ethanol, and flowed through a Dialon HP-20 (RTM) packed column (70mm x 100cm). Next, after removing water soluble components,</p>	

86-329949/50 YAMANOUCHI PHARM KK 24.04.85-JP-088235 (04.11.86) C12p-17/06 C12r-01/38 Prod. of genistein - by incubation of specified <i>Pseudomonas</i> strain sepd. from soil in liq. medium contg. nutrient C86-143202	B(6-A1, 12-G1B2, 12-G4C) D(5-C) 2 From are used. The bacteria are incubated in a liq. medium contg. carbon and nitrogen sources under aerobic conditions at 20-40°C for 50-150 hr. Genistein is collected from the medium by the usual methods, i.e. removal of bacterial cells by filtration or centrifugation, extn. from the resulting filtrate with an organic solvent, treatment by non-ionic adsorption, resin, pH adjustment, lyophilisation, evapn., etc. Thus obtd. genistein has the following physico-chemical properties: Mol. wt. (Mass Spectrum): 270 Molecular formula: C ₁₅ H ₁₀ O ₅ UV-absorption spectrum: λ_{max} = 263 nm ¹ H NMR-spectrum: CD ₃ OD (δ): 8.05 (1H, s) 7.37 (2H, d) 6.84 (2H, d) 6.34 (1H, d) 6.22 (1H, d) Solubility: Soluble in methyl alcohol and ethyl alcohol; hardly soluble in ethyl acetate, acetone and chloroform; insoluble in water, benzene and toluene. Colour reaction: Positive in potassium permanganate; negative in ninhydrin reaction and Dragendorff reaction. Property: Acidic. The above properties agree with those of synthetised genistein. (5 pPHDDwgNo0/0). J61247396-A
86-329949/50 B02 D16 YAMA 24.04.85 *J6 1247-396-A Prod. of genistein (4',5,7-trihydroxyisoflavone (1)) comprises incubating a strain of <i>Pseudomonas</i> capable of producing genistein and collecting genistein from the culture.  USE (1) has oestrogen activity and can inhibit the activity of tyrosine kinase, prod. of an oncogene. PROCEDURE <i>Pseudomonas</i> sp. YO-0170J, which can produce genistein, is sepd. from soil. This bacterium or mutants derived there-	

- (54) IMMUNO-SUPPRESSOR
(11) 62-106016 (A) (43) 16.5.1987 (19) JP
(21) Appl. No. 60-245308 (22) 1.11.1985
(71) YAMANOUCHI PHARMACEUT CO LTD(J)
(72) SHUNICHI WATANABE(3)
(51) Int. Cl. A61K31/35//C07D311/34

PURPOSE: To provide an immuno-suppressor containing a specific isoflavone compound as an active component, having low toxicity and excellent immuno-suppressing activity and useful for the remedy and the prevention of relapse of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, etc.

CONSTITUTION: The isoflavone compound of formula (R¹ is OH or methoxy; R² is H, carboxyl or ethoxycarbonyl) is used as an immuno-suppressing agent. Concrete examples of the compound are 5,7,4'-trihydroxyisoflavone, 5,7-dihydroxy-4'-methoxyisoflavone-2-carboxylic acid, etc. The compound of formula

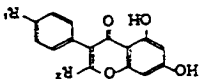


has excellent immuno-suppressing activity and is useful for the remedy and prevention of relapse of human autoimmune diseases such as chronic active hepatitis, osteoporosis, etc. It is administered orally or parenterally at a dose of usually 200~1,000mg/day.

- (54) ANTI-TUMOR AGENT
(11) 62-106017 (A) (43) 16.5.1987 (19) JP
(21) Appl. No. 60-245309 (22) 1.11.1985
(71) YAMANOUCHI PHARMACEUT CO LTD(J)
(72) HIROSHI OGAWARA(2)
(51) Int. Cl. A61K31/35//C07D311/34

PURPOSE: To provide an anti-tumor agent containing 5,7-dihydroxy-4'-substituted-isoflavone-2-carboxylic acid or its ethyl ester as an active component and having strong oncogene-originated tyrosine-specific phosphorilase-inhibiting activity.

CONSTITUTION: The 5,7-dihydroxy-4'-substituted-isoflavone-2-carboxylic acid of formula (R¹ is OH or methoxy; R² is carboxyl or ethoxycarbonyl) or its ethyl ester is used as an anti-tumor agent. The compound of formula 5,7-dihydroxy-4'-methoxyisoflavone-2-carboxylic acid. The compound of formula strongly inhibits oncogene-originated tyrosine-specific phosphorilase which is suspected to participate in the canceration of normal cell and the proliferation of cancer cell. Accordingly, the agent is useful for the prevention of carcinogenesis and remedy of cancer.



(54) WATER-BASED PREPARATION CONTAINING VITAMIN E IN HIGH CONCENTRATION

- (11) 62-106018 (A) (43) 16.5.1987 (19) JP

- (21) Appl. No. 60-246695 (22) 1.11.1985
(71) SANTEN PHARMACEUT CO LTD (72) JUNICHI IWAO(2)
(51) Int. Cl. A61K31/35, A61K9/10

PURPOSE: To obtain a water-based preparation containing vitamin E in high concentration and useful as a medicine such as ophthalmic solution, by using vitamin E or its fatty acid ester at a concentration essentially higher than the solubility in water at normal temperature and emulsifying the compound or dissolving the compound by the aid of a dissolution assistant.

CONSTITUTION: A water-based preparation containing homogeneously dissolved vitamin E in high concentration can be produced by adding a dissolution assistant (e.g. polysorbate 80, polyoxyethylene hardened castor oil, etc.) to a vitamin E compound (e.g. D/-α-tocopherol, d-α-tocopherol acetate, etc.). As an alternative, a water-based preparation containing emulsified vitamin E in high concentration is obtained by emulsifying a vitamin E compound with an emulsifier such as soybean lecithin. The water-based preparation has sufficiently high concentration (1~30%) to exhibit remedying effects such as antioxidation activity, oxygen-retaining activity, etc., and is applicable as an ophthalmic solution, etc.

(54) DRUG COATING MATERIAL

- (11) 2-67214 (A) (43) 7.3.1990 (19) JP
 (21) Appl. No. 63-218928 (22) 31.8.1988
 (71) YAMANOUCHI PHARMACEUT CO LTD (72) MUNETAKA HATTORI

PURPOSE: To obtain a drug coating composition, containing saccharides, calcium lactate and crystalline cellulose as essential ingredients, eliminating the need of a binder, excellent in strength and capable of withstanding change with time and providing a sugar-coated tablet.

CONSTITUTION: Calcium lactate in an amount of 0.5-10% and crystalline cellulose as a suspensible powder in an amount of 1-8% and saccharides (e.g. sucrose or glucose) providing a syrup solution in an amount of 40-70% are contained in a coating solution. Distilled water or an organic solvent is used as a solvent and coating is carried out by a conventional method to afford a coated drug. Although a normally blended binder, such as gelatin or gum arabic powder, is not required, a large amount of suspensible powder of talc is blended in the syrup to increase strength. Furthermore, the crystalline cellulose is blended to increase the resistance to peeling on the surface of a sugar coated tablet.

(54) ANTITUMOR AGENT

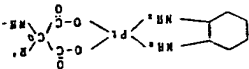
- (11) 2-67217 (A) (43) 7.3.1990 (19) JP
 (21) Appl. No. 63-219266 (22) 1.9.1988
 (71) TANABE SEIYAKU CO LTD (72) KENJI TSUJIHARA

NEW MATERIAL: A compound expressed by formula I (R¹ is H or alkyl; R² is H, lower alkyl, lower alkenyl, etc.; Alk is lower alkylene; X is carbonyl or sulfonyl; n is 1 or 2).

EXAMPLE: [2-{(N-(Chloroacetyl) glycy) amino} malonato] (trans -/-, 1, 2-diaminocyclohexanepiplatium(II)).

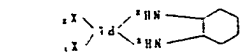
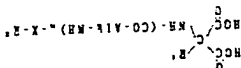
USE: Antitumor agent, having high water solubility and hardly exhibiting any nephrotoxicity.

PREPARATION: An amine compound expressed by formula II (h is 0, 1 or 2 without exceeding n) or ester thereof is reacted with an acid compound organic III according to a conventional method for synthesizing peptides and, as necessary, hydrolyzed to provide a 2-(substituted amino)malonic acid expressed by formula IV or salt thereof (e.g. alkaline metal or silver salt), which is then reacted with a 1,2-diaminocyclohexanepiplatium complex expressed by formula V (X¹ and X² are reactive residue) to afford the compound expressed by formula I.



(R¹ or R²) (CO-Alk-NH) or (CO-Alk-NH)₂ (R¹ is H or alkyl; R² is H, lower alkyl, lower alkenyl, etc.; Alk is lower alkylene; X is carbonyl or sulfonyl; n is 1 or 2).

HO-(CO-Alk-NH) or (CO-Alk-NH)₂ (R¹ is H or alkyl; R² is H, lower alkyl, lower alkenyl, etc.; Alk is lower alkylene; X is carbonyl or sulfonyl; n is 1 or 2).



(54) VIRUS GENOME INACTIVATOR

- (11) 2-67218 (A) (43) 7.3.1990 (19) JP
 (21) Appl. No. 63-217427 (22) 31.8.1988
 (71) NAGAKURA SEIYAKU K.K. (72) TAKAO KIJIMA

- (51) Int. Cl. A61K31/045, A61K31/36, A61K31/70, A61K35/78, C07C35/44, C07D311/36

PURPOSE: To obtain a virus-genome inactivator containing a specific compound, such as afromosin or formononetin, as an active ingredient and effective in antiviral, carcinostatic use and preventing cancer.

CONSTITUTION: A virus-genome inactivator containing one or two or more of compounds, expressed by formula I, i.e., afromosin (R¹ is OH; R² is OCH₃), formononetin (R¹ is OH; R² is H), ononin (R¹ is O-glucose; R² is H), wistin (R¹ is O-glucose; R² is O-acetyl; R³ is O-acetyl; R⁴ is O-glucose; R⁵ is O-glucose; R⁶ is O-glucose; R⁷ is O-glucose; R⁸ is O-glucose; R⁹ is O-glucose; R¹⁰ is O-glucose; R¹¹ is O-glucose; R¹² is O-glucose; R¹³ is O-glucose; R¹⁴ is O-glucose; R¹⁵ is O-glucose; R¹⁶ is O-glucose; R¹⁷ is O-glucose; R¹⁸ is O-glucose; R¹⁹ is O-glucose; R²⁰ is O-glucose; R²¹ is O-glucose; R²² is O-glucose; R²³ is O-glucose; R²⁴ is O-glucose; R²⁵ is O-glucose; R²⁶ is O-glucose; R²⁷ is O-glucose; R²⁸ is O-glucose; R²⁹ is O-glucose; R³⁰ is O-glucose; R³¹ is O-glucose; R³² is O-glucose; R³³ is O-glucose; R³⁴ is O-glucose; R³⁵ is O-glucose; R³⁶ is O-glucose; R³⁷ is O-glucose; R³⁸ is O-glucose; R³⁹ is O-glucose; R⁴⁰ is O-glucose; R⁴¹ is O-glucose; R⁴² is O-glucose; R⁴³ is O-glucose; R⁴⁴ is O-glucose; 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(64) LIPOSOME PREPARATION AND PRODUCTION THEREOF
(21) Appl. No. 63-315080 (22) 15.12.1988
(43) 20.6.1990 (19) JP
(71) NIPPON OIL & FATS CO LTD(1) (72) HIDEHIKO HIRINO(4)
(51) Int. Cl. A61K35/78, A61K31/35, A61K31/70, A61K35/78

PURPOSE: To obtain a liposome preparation having excellent stability by treating a flavonoid with an alkaline solution and converting into liposome.
CONSTITUTION: A phospholipid or a mixture of phospholipid and cholesterol is formed in the form of a thin film by reverse-phase evaporation process. A solution of free flavonoid dissolved in an alkaline solution having pH of ≥ 10 is added to the thin film and uniformized by ultrasonic process to obtain a stable emulsion. The objective liposome preparation containing a flavonoid is produced by treating the emulsion with an extruder. The preparation can be infused into a body by intravenous or transperitoneal infusion to keep high flavonoid concentration in the body and is effective as a platelet coagulation inhibitor, a vasodilator, an antihistaminic, an anti-inflammatory, an antispasmodic, an estrone-like agent and a scavenger for free radical, etc.

(64) CALCITONIN-CONTAINING INJECTION
(21) 2-160725 (A) (43) 20.6.1990 (19) JP
(21) Appl. No. 63-314095 (22) 13.12.1988
(71) MITSUBISHI KASEI CORP (72) YOSHINORI MATSUOKA(2)
(51) Int. Cl. A61K37/30, A61K9/08, A61K37/30

PURPOSE: To obtain an analgesic injection containing avian calcitonin and stable over a long period by suppressing the concentration of a pH modifier to be added for stabilization to a low level and adjusting the pH of the injection to weakly acidic state.
CONSTITUTION: The objective avian calcitonin-containing injection contains a pH modifier at a concentration of ≤ 10 mM (m-mol/l), preferably 2-10 mM (m-mol/l) and has weakly acidic pH (especially 5.0-7.0). The pH modifier is citric acid, acetic acid, phosphoric acid, tartaric acid, lactic acid, their sodium salt or potassium salt or sodium hydroxide, etc., and is used singly or as a proper combination of two or more compounds.

(64) REMOVAL OF TRACE SULFUR COMPOUND IN HYDROCARBON
(11) 2-160728 (A) (43) 20.6.1990 (19) JP
(21) Appl. No. 63-317111 (22) 15.12.1988
(71) KAWASAKI STEEL CORP (72) YUKIO ASAMI(2)
(51) Int. Cl. C07C7/12, C10G29/16

PURPOSE: To remove trace sulfur compound in an aromatic hydrocarbon by contacting with a desulfurizing agent composed mainly of copper oxide having specific BET surface area and pore volume and reduced at a specific temperature in hydrogen stream.
CONSTITUTION: An aromatic hydrocarbon is made to contact with a desulfurizing agent composed mainly of copper oxide at 150-179°C to remove trace sulfur compound contained in the hydrocarbon. The desulfurizing agent is composed mainly of copper oxide, has a BET surface area of 10-150 m²/g and a pore volume of ≤ 0.1 cc/g and is produced by supporting 20-60 wt.% of copper oxide on a carrier and reducing the oxide at 150-200°C in hydrogen stream. The desulfurization can be carried out in high efficiency in the absence of hydrogen.

FLAVONOIDS AS DNA TOPOISOMERASE ANTAGONISTS AND POISONS: STRUCTURE-ACTIVITY RELATIONSHIPS

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ABSTRACT.—Selected flavonoids were tested for their ability to inhibit the catalytic activity of DNA topoisomerase (topo) I and II. Myricetin, quercetin, fisetin, and morin were found to inhibit both enzymes, while phloretin, kaempferol, and 4',6,7-trihydroxyisoflavone inhibited topo II without inhibiting topo I. Flavonoids demonstrating potent topo I and II inhibition required hydroxyl group substitution at the C-3, C-7, C-3', and C-4' positions and also required a keto group at C-4. Additional B-ring hydroxylation enhanced flavonoid topo I inhibitory action. A C-2,C-3 double bond was also required, but when the A ring is opened, the requirement for the double bond was eliminated. Genistein has been previously reported to stabilize the covalent topo II-DNA cleavage complex and thus function as a topo II poison. All flavonoids were tested for their ability to stabilize the cleavage complex between topo I or topo II and DNA. None of the agents stabilized the topo I-DNA cleavage complex, but prunetin, quercetin, kaempferol, and apigenin stabilized the topo II DNA-complex. Competition experiments have shown that genistein-induced topo II-mediated DNA cleavage can be inhibited by myricetin, suggesting that both types of inhibitors (antagonists and poisons) interact with the same functional domain of their target enzyme. These results are of use for the selection of flavonoids that can inhibit specific topoisomerases at specific stages of the topoisomerization reaction.

Several plant-derived flavonoids have been previously reported to inhibit certain regulatory enzymes including protein kinase C (1), reverse transcriptase (2), and DNA topoisomerase (topo) II (3,4). With the exception of genistein, the specific effects of these flavonoids on the two main types of eukaryotic topoisomerases (topo I and topo II) have not been determined. Drugs targeting either topo I or topo II have applications in cancer chemotherapy, but the range of applicable tumors depends on the type of topoisomerase targeted and on their mode of action. For example, the topo I-targeting drugs camptothecin and topotecan, are effective against slow-growing tumors that contain the same levels of topo I as more rapidly growing tumors (5). In contrast, the topo II-targeting etoposide (VP-16) and teniposide (VM-26) are more useful in rapidly proliferating carcinomas expressing high levels of topo II. These clinical considerations necessitate the precise identification of the enzymatic targets of the flavonoids and other compounds that inhibit DNA topoisomerases as well as a clear understanding of their mode of action.

In eukaryotes, DNA topoisomerases are involved in the processes of DNA replication, transcription, and recombination (6), and also play a key role during cell

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proliferation and differentiation (7–12). Inhibitors of eukaryotic topoisomerases, depending on the stage of the catalytic cycle they inhibit, can be distinguished into two classes. In the first class belong cytotoxic agents known as topoisomerase poisons (13), which, by preventing the religation step of the reaction, stabilize the covalent enzyme-DNA complex known as the cleavage complex (14,15). Teniposide, etoposide, ellipticine, doxorubicin, and *m*-AMSA are some representative topo II poisons. In the second class belong agents that do not stabilize the covalent enzyme-DNA complex, but rather prevent formation of this complex and consequently enzymatic turnover; these have been referred to as topoisomerase antagonists because they oppose both the normal topoisomerase strand-passing reaction as well as the action of the poisons (16). Novobiocin (17), merbarone (18), aclarubicin (19), fostriecin (20), *bis*-2,6-dioxopiperazine derivatives (21), and gossypol (22) are some representative topo II antagonists. Although topo I and II poisons are more commonly used as chemotherapeutic agents (11–13,23), some topo II antagonists that exhibit antitumor effects in animal studies have been reported (24–27).

In the present study, 20 representative flavonoids have been evaluated using specific assays, for their ability to inhibit topo I and topo II activities and to enhance DNA-strand breakage. Structural alterations that drastically change an agent's effectiveness or mode of action have been identified. We report herein that topo II-inhibiting flavonoids can function as topo II poisons, antagonists, or both, depending on the position of hydroxyl groups in the A and B rings of the molecule.

RESULTS AND DISCUSSION

The effect of various flavonoids on the catalytic activity of topo I and topo II was evaluated using the relaxation and unknotting assays, respectively. Initially, compounds were evaluated at a concentration of 100 $\mu\text{g/ml}$. Agents not showing activity at 100 $\mu\text{g/ml}$ were considered ineffective and were not tested further. Agents showing inhibition at 100 $\mu\text{g/ml}$, were further tested in the 1- through 100- $\mu\text{g/ml}$ range until the minimal concentration necessary to inhibit 50% of the topo I or topo II catalytic activity (IC_{50}) was determined. An example of this approach is shown in Figure 1. In the initial test, quercetin inhibited both topo I and topo II at 100 $\mu\text{g/ml}$. This compound was then further evaluated at lower concentrations as shown in Figure 1A. When the photographic negative was scanned and the densitometric data plotted as shown in Figure 1C, the IC_{50} value was found to be 10 $\mu\text{g/ml}$. By averaging the IC_{50} values of three to four such experiments, mean values shown in Tables 1 and 2 were obtained. A similar approach was used for determining the mean IC_{50} values of quercetin for topo II-catalytic activity, except that the photographic negatives of unknotting assays (shown in Figure 1B) were scanned, and the density of the unknotted band was integrated and plotted as illustrated in Figure 1D.

In this manner, the ability of representative flavones to inhibit the catalytic activities of topo I and II was determined, and these results are summarized in Table 1. The flavone derivatives myricetin, quercetin, fisetin, and morin inhibited both topo I-relaxing activity and topo II-unknotting activity. Kaempferol and phloretin inhibited only topo II-unknotting activity. Effective topo II-inhibiting flavones have in common the obligatory C-4 keto group and hydroxyl group substitutions at C-3, C-7, and C-4'. Effective topo I inhibitors exhibited the same structural parameters as topo II inhibitors, with the additional requirement of another hydroxyl group in the B-ring. When this additional group was adjacent to the C-4' position the flavonoid was more effective in inhibiting topo I. *d*-Catechin, a flavanol with the same hydroxyl group substitutions as the flavone quercetin, did not inhibit either topo I or topo II. Rutin, which also has the same hydroxyl group substitutions as quercetin, with the exception of being a C-3

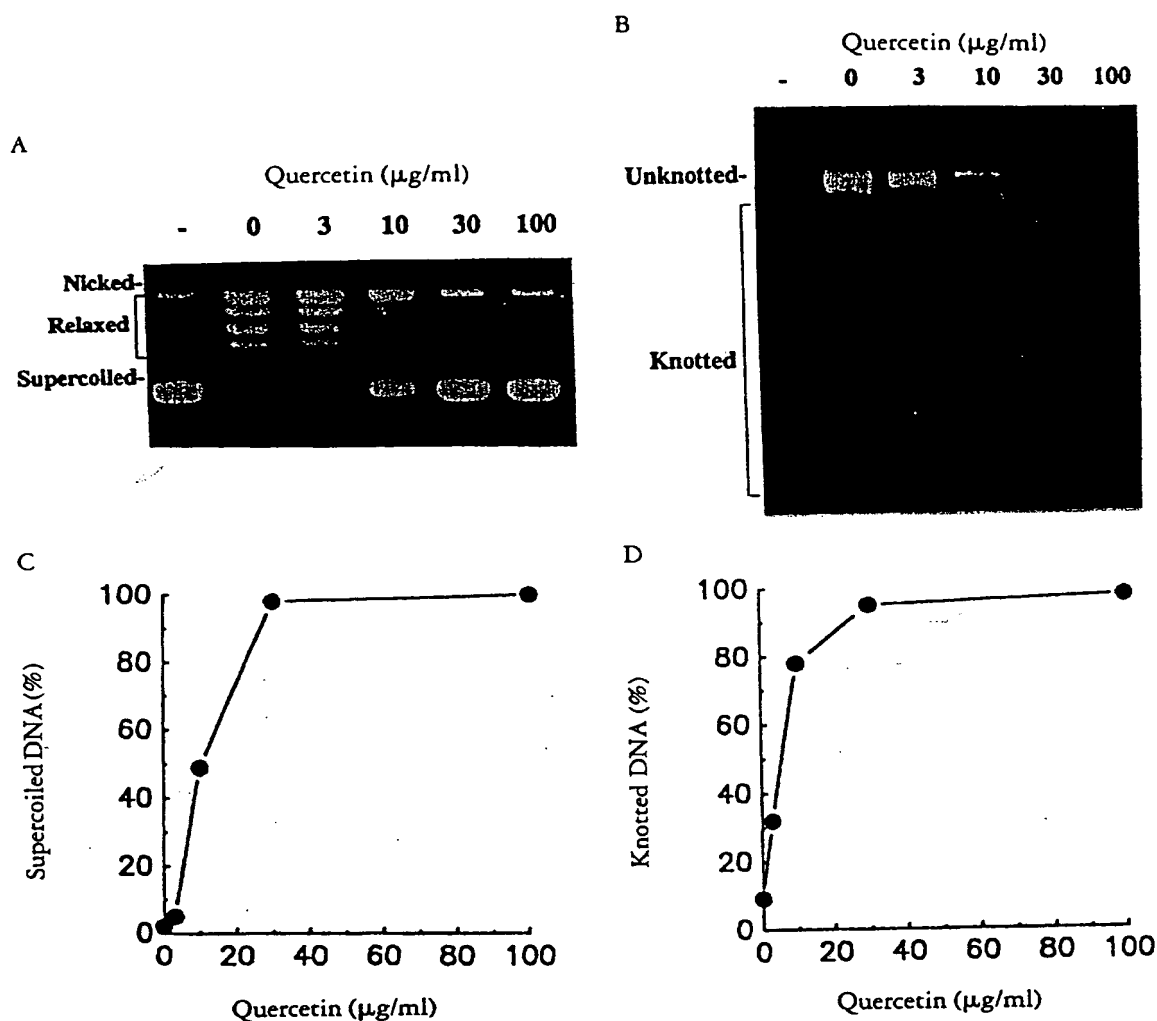


FIGURE 1. Agarose gel assays for determining the effect of quercetin on topo I (A) and topo II activities (B). Two units of purified topo I or topo II were incubated, under the conditions described in the Experimental, with increasing concentrations of quercetin as shown in the top of panels A and B. The amount of the reaction substrate, evaluated by scanning densitometrically the supercoiled form of pUC8 DNA for topo I (panel C), or the unknotted form of P4 DNA for topo II (D), is plotted against quercetin concentration. The IC_{50} value was determined by extrapolating the concentration of quercetin required to prevent conversion of 50% of the substrate to the reaction product. This is a representative experiment; mean IC_{50} values were determined in this manner from a total of three independent experiments.

glycoside, lacked topoisomerase inhibitory activity. Galangin was ineffective in inhibiting topoisomerases, apparently because it lacks hydroxyl groups in the B ring. Flavone and all monohydroxyflavones were found in this study to be ineffective as enzyme inhibitors.

From the five isoflavones tested, only genistein (4',5,7-trihydroxyisoflavone) and 4',6,7-trihydroxyisoflavone inhibited topo II-unknotting activity (Table 2). Genistein has been previously shown to inhibit topo II-unknotting activity, while it is unable to inhibit topo I-relaxing activity at concentrations lower than 1 mM (28,29). In the present study, genistein was used as a positive control. Daidzein, which differs from genistein in that it has no hydroxyl group at the C-5 position, did not inhibit topo II- (or topo I-) unknotting activity. Prunetin, with a C-7 methoxyl group was also ineffective in this assay. From these results, it was concluded that hydroxyl groups at C-

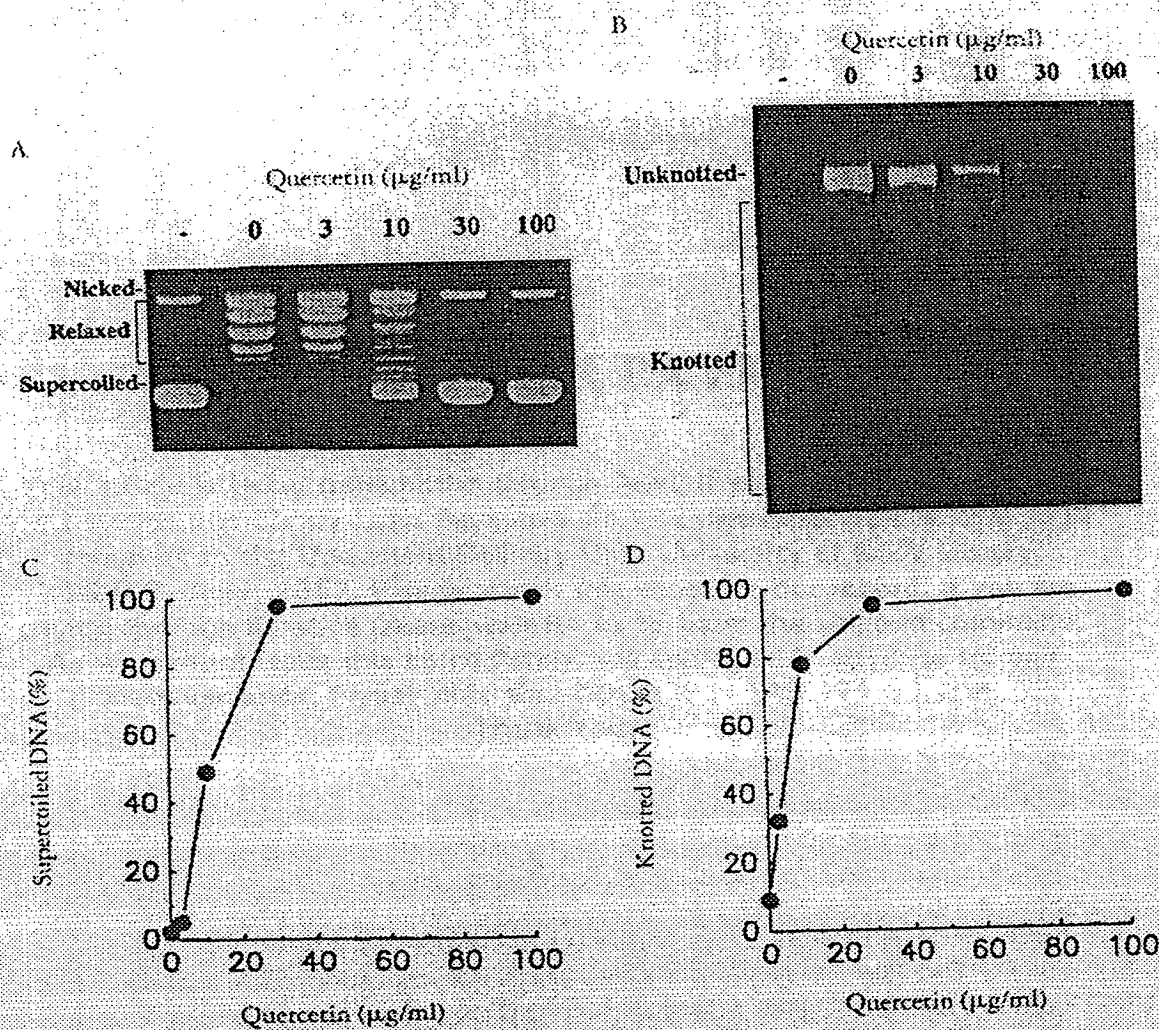


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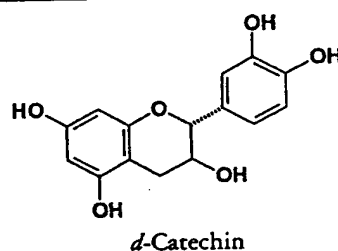
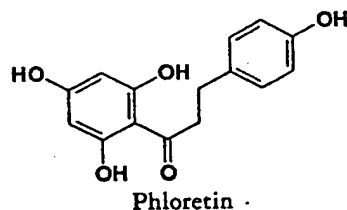
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TABLE 1. Effect of Flavones, a Dihydrochalcone, a Flavanol, and Derivatives on Topo I-Relaxing and Topo II-Unknotting Activities.

Compound	Position of Hydroxyl Substitution		Mean ^a IC ₅₀ (μg/ml)	
	A ring	B ring	Topo I	Topo II

Flavone			— ^b	— ^b
Myricetin	3 5 7	3' 4' 5'	11.9	11.9
Quercetin	3 5 7	3' 4'	12.8	6.9
Fisetin	3 5 7	3' 4'	20.6	8.2
Morin	3 5 7	2' 4'	42.1	40.8
Kaempferol	3 5 7	4'	— ^b	8.1
Apigenin	5 7	4'	— ^b	— ^b
Rutin ^c	5 7	3' 4'	— ^b	— ^b
Galangin	3 5 7		— ^b	— ^b
3-OH Flavone	3		— ^b	— ^b
5-OH Flavone	5		— ^b	— ^b
6-OH Flavone	6		— ^b	— ^b
7-OH Flavone	7		— ^b	— ^b



Phloretin	3 5 7	4'	— ^b	46.1 ^b
d-Catechin	3 5 7	3' 4'	— ^b	— ^b

^aValues represent the mean from at least three independent experiments, the variance was less than 25% of the mean value.

^bNo effect at up to 100 μg/ml final concentration.

^cContains a rutinoside sugar unit attached to C-3.

5, C-7, and C-5' are required for the inhibition of topo II-unknotting activity by isoflavonoids. However, C-6 instead of C-5 hydroxyl substitution is permissible. None of these isoflavonoids inhibited topo I at concentrations below 100 μg/ml.

To further characterize the stage of the topoisomerization reaction initially blocked by the agent, we used a linearization assay. Topo II poisons stabilizing the cleavage complex can be evaluated using the pUC8 plasmid linearization assay. The production of linear plasmid DNA by an agent in the presence of topo II and after proteinase-K SDS digestion indicates the ability of the agent to stabilize the cleavage complex (Figure 2). Using this, or other similar assays, genistein has been shown to stabilize the cleavage complex (3, 4, 28, 29). The flavonoids listed in Tables 1 and 2 were evaluated for their ability to induce DNA-breakage in the presence of topo II. Positive agents are listed in Table 3; quercetin, kaempferol, apigenin, and prunetin enhanced by at least 60% the

TABLE 2. Effect of Isoflavones on Topo I-relaxing and Topo II-Unknotting Activities.

Compound	Position of Hydroxyl Substitution		Mean ^a IC ₅₀ (μg/ml)	
	A ring	B ring	Topo I	Topo II

Genistein	5	7	4'	— ^b	30
Daidzein		7	4'	— ^b	— ^b
3',4',7-Trihydroxyisoflavone . . .		7	3' 4'	— ^b	— ^b
4',6,7-Trihydroxyisoflavone . . .	6	7	4'	— ^b	43.7
Prunetin	5	7-OMe	4'	— ^b	— ^b

^aValues represent the mean from at least three independent experiments; the variance was less than 25% of the mean value.

^bNo effect up to 100 μg/ml final concentration.

topo II-mediated DNA cleavage as determined by increases in the linear form of plasmid DNA in the presence of topo II, but these agents were considerably less potent than genistein. In two experiments, fisetin, morin, 5-hydroxyflavone, and 6-hydroxyflavone weakly enhanced the cleavage complex (by less than 50%). These agents, however, were considered marginally effective and are not listed in Table 3. None of the effective agents produced linear DNA in the absence of the enzyme, indicating that the effect is topo II-mediated.

It is currently unknown whether flavonoid antagonists of topo II bind on the same site as flavonoid topo II poisons. To address this issue, we evaluated the effect of myricetin, which is a strong inhibitor of topo II-unknotting activity (IC₅₀ 11.9 μg/ml), on the DNA cleavage enhancing-effect of genistein. The results are shown in Figure 2. Genistein, at concentrations of 100 μg/ml (lane 3) and 30 μg/ml (lane 6) effectively produced linear DNA (form III), in the presence of topo II. Myricetin, at concentrations ranging from 3 to 100 μg/ml, did not produce linear DNA. Only the highest myricetin concentration is shown in this figure (lane 4). Furthermore, myricetin prevented

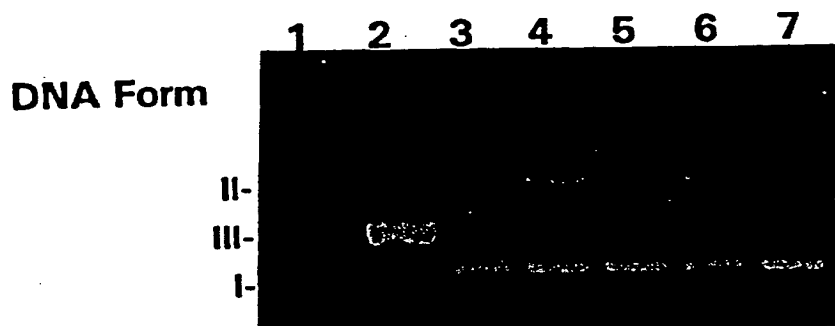


FIGURE 2. Effects of myricetin on topo II-mediated cleavage of plasmid pUC8 DNA. Lane 1, control (no drug); lane 2, linear pUC8 (marker for form III-DNA); lane 3, genistein (100 μg/ml); lane 4, myricetin (100 μg/ml); lane 5, genistein (100 μg/ml) plus myricetin (100 μg/ml); lane 6, genistein (30 μg/ml); lane 7, genistein (30 μg/ml) plus myricetin (30 μg/ml). All reactions (except lane 3) containing 10 units of purified human topo II were performed as described in the Experimental under "Plasmid Linearization Assay."

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	A ring	B ring	Topo I	Topo II

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Daidzein		7	4'	— ^b
3',4',7-Trihydroxyisoflavone		7	3', 4'	— ^b
4',6,7-Trihydroxyisoflavone	6	7	4'	43.7
Prunetin	5	7-OMe	4'	— ^b

^aValues represent the mean from at least three independent experiments; the variance was less than 25% of the mean value.

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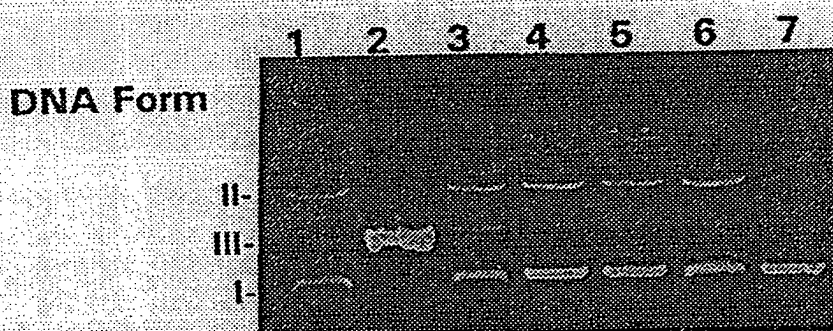


FIGURE 2. Effects of myricetin on topo II-mediated cleavage of plasmid pUC8 DNA. Lane 1, control (no drug); lane 2, linear pUC8 (marker for form III-DNA); lane 3, genistein (100 μg/ml); lane 4, myricetin (100 μg/ml); lane 5, genistein (100 μg/ml) plus myricetin (100 μg/ml); lane 6, genistein (30 μg/ml); lane 7, genistein (30 μg/ml) plus myricetin (30 μg/ml). All reactions (except lane 3) containing 10 units of purified human topo II were performed as described in the Experimental under "Plasmid Linearization Assay."

TABLE 3. Flavonoids Effective in Enhancing Topo II-Mediated DNA Cleavage.

Compound ^a	Linear DNA ^b (% of total DNA)	Linear DNA (times control value)
None (control)	5.3	1
Quercetin	11.5	2.2
Kaempferol	8.3	1.6
Apigenin	8.7	1.6
Genistein	16.2	3.1
Prunetin	9.2	1.7

^aAll flavonoids were tested, as shown in Figure 2, at a final concentration of 100 μ g/ml.

^bValues are the means from at least triplicate experiments; the variance was less than 25% of the mean value.

genistein's DNA cleavage-promoting action as determined by the absence of linear DNA in lanes 5 and 7. Myricetin antagonized the genistein-induced DNA breakage only when it was provided at the same concentration as the isoflavone. Myricetin, when introduced at lower concentrations than genistein, was only partially effective and when genistein was at 10-fold excess myricetin became ineffective in reducing the linear form of DNA (data not shown). This antagonistic action of myricetin was not limited to genistein, since it also prevented the DNA cleavage action of prunetin (data not shown). One possible explanation of these results is that flavonoids inhibiting different stages of the topoisomerization reaction may bind on the same site of the enzyme or the DNA enzyme-complex.

The C-4 carbonyl group was found to be essential for the inhibition of both topo I and topo II activities. This is apparent by comparing the active quercetin with the inactive *d*-catechin. Comparison of these two compounds also suggested that the C-2, C-3 double bond in the pyrone ring is essential for inhibiting both topoisomerases. This requirement, however, is not necessary when the A ring is cleaved, as in the case of phloretin. Hydroxyl group substitution in the A and B rings provides additional specificity. These critical structural observations are discussed below.

Myricetin, quercetin, and fisetin were the most potent inhibitors of topo I and topo II catalytic activities among the flavonoids investigated; these have in common hydroxyl group substitutions at positions C-3, C-7, C-3', and C-4'. The inability of rutin (in which the C-3 position is glycosylated) to inhibit either one of the two enzymes studied further demonstrates the requirement for hydroxyl group substitution at C-3. Phloretin was an apparent exception to this structural requirement, although this is based on a dihydrochalcone rather than a flavone structure. The three flavones, quercetin, kaempferol, and apigenin that functioned as topo II poisons have as a common characteristic C-5, C-7, and C-4' hydroxyl group substitutions.

Similar but not identical structural requirements have been reported for flavonoid inhibitors of other enzymes. For example C-7, C-3', and C-4' hydroxyl group substitution was required for the inhibition of protein kinase C, while C-3, C-5, C-7, C-3', and C-4' hydroxylation was required for the inhibition of reverse transcriptase (1,2). These data suggest that the above enzymes may contain a common amino acid sequence serving as the flavonoid recognition site.

It has been postulated previously that the ATP binding domain of topo II may also serve as the binding site for genistein (29). The ability of quercetin and genistein to inhibit the ATPase component of the topoisomerization reaction (30) provides some support to this hypothesis. In addition, the demonstration of antagonism between myricetin and genistein provides further support. Based on the observation that

genistein-induced (topo II-mediated) DNA breakage is prevented by equimolar concentrations of myricetin, we propose that the two flavonoids bind on the same site of the target enzyme; different stages of the topoisomerization reaction, however, are blocked due to spatial differences between the two flavonoids. Genistein, which is planar, stabilizes the cleavable complex, while the nonplanar myricetin prevents enzymatic turnover.

An alternative explanation of the above observations requires the flavonoids to exert their effects non-specifically, that is, through intercalation into DNA. Three lines of evidence oppose this possibility. First, Yamashita *et al.* reported that neither myricetin nor genistein intercalate into pBR322 DNA even at concentrations of 250 μ M (3). Second, no correlation between DNA intercalation and induction of DNA breakage has been shown in any studies with flavonoids or other topo II-targeting agents (3,4). Third, if the effect were non-specific, one would expect myricetin to also inhibit camptothecin-mediated topo I nicking. We have found that myricetin had no effect on camptothecin-induced topo I-mediated DNA breakage (unpublished results). Our data, in conjunction with those from the above studies, suggest that the competition between genistein and myricetin is due to the binding of drugs with comparable affinity for the same pharmacophore (topo II) and not due to nonspecific intercalation of myricetin into DNA. Thus, flavonoid antagonists may bind with a spatial orientation that neither interferes with the DNA cleavage/religation equilibrium, nor opposes the DNA strand-passage step of the reaction (31); but rather they inhibit enzymatic turnover through a mechanism requiring ATP hydrolysis. Flavonoid poisons, on the other hand, because of a different spatial arrangement, may stabilize the (normally transient) DNA-enzyme complex and favor the DNA cleavage component of the reaction. We have demonstrated here that the effects of flavonoids on the topoisomerization reaction are varied, and, depending on minor structural alteration, these agents can function either as topo II antagonists, or as topo II poisons.

EXPERIMENTAL

CHEMICALS AND REAGENTS.—The sources of the chemicals used in the present study were as follows: apigenin, kaempferol, quercetin, myricetin, *d*-catechin, phloretin, and morin (Sigma Chemical Co., St. Louis, MO); flavone, 3-hydroxyflavone, galangin, fisetin, and rutin (Aldrich Chemical Co., Milwaukee, WI); 5-hydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, genistein, and daidzein (Indofine Chemical Co., Somerville, NJ); *m*-AMSA from the National Cancer Institute; etoposide and teniposide (Bristol-Myers Squibb Co., Wallingford, CT). Topo I was purchased from Gibco-BRL, Gaithersburg, MD, and human topo II was purchased from Topogen, Inc., Columbus, OH. Stock solutions of all flavonoids, etoposide, and teniposide were prepared in DMSO; *m*-AMSA was dissolved in EtOH.

TOPOISOMERASE I RELAXATION ASSAY.—For the determination of topoisomerase (topo) I catalytic activity, pUC8 DNA was used as the substrate in a reaction volume of 20 μ l containing the following: 50 mM Tris-Cl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 30 μ g/ml bovine serum albumin and 2 units of purified calf thymus topo I. The appropriate inhibitor was added when necessary, and the reaction was started by the addition of the enzyme. Reactions were carried out at 37° for 30 min. Gel electrophoresis was performed at 4 V/cm for 5 h in Tris-borate-EDTA buffer. For the quantitative determination of topo I activity, photographic negatives were densitometrically scanned (Hoefer Scientific Instruments GS300 Scanning Densitometer). The area representing supercoiled DNA, migrating as a single band at the bottom of the gel, was determined. The concentration of the inhibitor at which it prevented 50% of the substrate (supercoiled DNA) from being converted into the reaction product (relaxed DNA) was determined by plotting the data as shown in Figure 1. By averaging three to four such experiments, the IC₅₀ values were determined.

TOPOISOMERASE II P4 UNKNOTTING ASSAY.—For the determination of topoisomerase (topo) II catalytic activity, knotted DNA that had been isolated from the tailless capsids of the bacteriophage P4 *Viral* *dello* was used as the substrate, basically as described by Liu *et al.* (32), but with some modifications. Reaction mixtures contained 50 mM Tris-Cl, pH 8.0, 100 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM EDTA, 40 μ g/ml bovine serum albumin (nuclease free), and 1 mM ATP. The appropriate inhibitor was added, when

necessary, followed by the addition of 2 units of human topo II. Reactions of 20 μ l total volume were started by the addition of 0.6 μ g of knotted DNA. Reactions were terminated by the addition of 5 μ l of a stop solution containing 5% SDS, 50 mM EDTA, 25% Ficoll, and 0.05 mg/ml bromophenol blue. Samples were loaded on 0.8% agarose gels and electrophoresed at 4 V/cm for 5 h in Tris-borate-EDTA buffer. Gels were stained in 1 μ g/ml ethidium bromide, destained, and photographed over a uv light source. For the quantitative determination of topo II activity, photographic negatives were densitometrically scanned. Unknotted DNA, migrating as a single band at the top of the gel, was measured in this manner. The concentration of the inhibitor preventing 50% of the substrate (knotted DNA) from being converted into the reaction product (unknotted DNA) was determined from a standard curve (Figure 1). By averaging three to four such experiments, the IC_{50} values were determined.

PLASMID LINEARIZATION ASSAY.—Topo II-targeting agents having the ability to enhance topo II-mediated DNA cleavage were screened using the linearization assay under the reaction conditions provided by the supplier of the enzyme (Topogen, Inc). Briefly, 20- μ l reaction mixtures contained 30 mM tris-Cl, pH 7.6, 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$, 60 mM NaCl, 1 μ l of the test agent if necessary, 0.3 μ g of pUC8 DNA, and 10 units of human topo II (added last). After a 30-min incubation at 37°, SDS-proteinase K was added and following a 15-min incubation at 37°, samples were extracted with $CHCl_3$ and electrophoresed on a 1% agarose gel containing 0.5 μ g/ml ethidium bromide. Gels were photographed, and photographic negatives were scanned using a Hoefer GS300 scanning densitometer. After integration of the three bands, linear DNA was expressed as percentage of total DNA.

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Short Communication

Antioxidative Stability of Tempeh

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Antioxidant activities of 6,7,4'-trihydroxyisoflavone and the compound 0.58 substance (Rf value 0.58 on Silicagel TLC with cyclohexane 9 : ethylether 1) isolated from tempeh were measured by oxygen absorption. Both are effective in preventing consumption of dissolved oxygen emulsified in safflower oil.

In response to the short communication on Tempeh oil antioxidant (1) by Stahl and Sims (1), it is necessary to report our further experimental work on 6,7,4'-trihydroxyisoflavone [a new isoflavone isolated from tempeh (2), and chemically synthesized for the study on its antioxidative activities (3)] and the other "0.58 substance" (Rf value, 0.58 on Silicagel TLC with developing solvent, cyclohexane 9 : ethylether 1) (4). The antioxidative and antihemolytic effects of 6,7,4'-trihydroxyisoflavone on vitamin A and sodium linoleate in aqueous solution at pH 7 have been reported (3). Later studies using the rapid method for evaluation of antioxidation by Yagi (5) not previously published are presented here.

EXPERIMENTAL PROCEDURES

According to the method described by Yagi (5), changes in oxygen content in a reaction mixture were measured with the Beckman Oxygen Analyzer (Model 777) attached to a recorder (Hitachi OPD 33) and connected to a sensor. The reaction mixture, which contained 10 g safflower oil, 100 ml water and one ml Tween 20, was blended for emulsification. Measurements were made immediately after addition of one ml of a test solution and one ml of a catalyst: 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ or 0.02% hemin.

RESULTS

Antioxidant activities of 6,7,4'-trihydroxyisoflavone measured by oxygen absorption method. As shown in Figure 1, 100 μg or 50 μg of 6,7,4'-trihydroxyisoflavone/40 ml emulsified solution of safflower oil (2.2 g) in the presence of the metal catalyst gave almost complete protection against oxygen consumption for the 10-min test period. In the control, almost 100% of the oxygen was consumed in this time.

Antioxidant activities of unknown compound (0.58 substance) from Tempeh by AOM. Results of the experiment on the 0.58 substance isolated from tempeh oil are shown in Figure 2. As seen there, 160 μg of the 0.58 substance/40 ml emulsified solution of safflower oil (2.2 g) protected against about 85% of dissolved oxygen consumption within several min, compared with 50% in the control in the presence of metal catalyst. However, 40 μg of the 0.58 substance using 0.02% hemin as catalyst gave little protection and was no more effective than 10 μg of BHT.

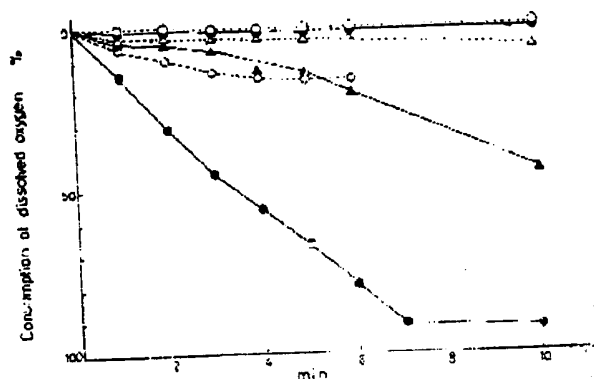


FIG. 1. Antioxidant activities of 6,7,4'-trihydroxyisoflavone (F2) measured by the oxygen absorption method. \bigcirc — \bigcirc , added F2, 100 $\mu\text{g}/40$ ml (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); \bullet — \bullet , added F2, 50 $\mu\text{g}/40$ ml (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); Δ — Δ , added F2, 25 $\mu\text{g}/40$ ml (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); \triangle — \triangle , added F2, 12 $\mu\text{g}/40$ ml (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); \bigcirc — \bigcirc , added F2, 100 $\mu\text{g}/40$ ml (catalyst, 0.02% Hemin), and \bullet — \bullet , control (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$).

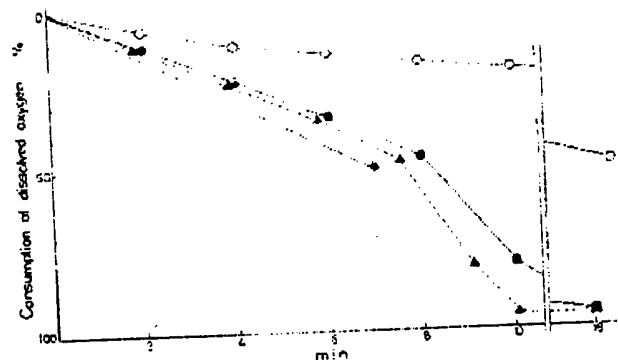


FIG. 2. Antioxidant activities of 0.58 substance measured by the oxygen absorption method. \bigcirc — \bigcirc , added 160 $\mu\text{g}/40$ ml 0.58 substance (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); \bullet — \bullet , added 40 $\mu\text{g}/40$ ml 0.58 substance (catalyst, 0.02% Hemin); Δ — Δ , added 10 $\mu\text{g}/40$ ml BHT (catalyst, 0.02% Hemin), and \bullet — \bullet , control (catalyst, 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$).

DISCUSSION

Stahl and Sims (1) emphasized that fatty acids liberated from soybeans during the fermentation of tempeh promote rapid decomposition of peroxide. Therefore, peroxide values determined during storage are a poor index of oxidation rate in oils high in FFA. From the

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results of oxygen absorption rates measured on oil at 50 C following the method of Bishov and Henick (6), Stahl and Sims (1) pointed out that their data clearly support the conclusion that the report by György was in error.

Stahl and Sims (1) did not mention our work of 1968 (3), in which the results of antioxidant activities in vitro and in vivo of 6,7,4'-trihydroxyisoflavone were reported together with discussion. The reference on the isolation of 6,7,4'-trihydroxyisoflavone is György et al. (2) and for the test of protection of peroxide development on the addition of as little as 10% tempeh oil to soybean oil is György et al. (7) and not György et al. (2) mentioned by them (1).

As mentioned by Yagi (5), antioxidants are classified in three types, according to the mechanism of action: chain breakers (or free radical inhibitors); peroxide decomposers, and metal inactivators. Flavonoids also were reported to act as chain breakers as well as metal inactivators. Chelated quercetin with cupric ion showed considerable loss of antioxidant activity when measured by the AOM test.

Our results using the AOM test clearly indicated that 6,7,4'-trihydroxyisoflavone and the 0.58 substance protected well against oxygen consumption for about a

10-min test period. Other reasons for stability of tempeh and mechanisms for its stability should be studied further.

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Isoflavonoids as Inhibitors of Lipid Peroxidation and Quenchers of Singlet Oxygen

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Flavonoids are a large group of plant polyphenols known to exhibit versatile antioxidant properties and a number of biological effects (1). Isoflavonoids have properties in common with the flavonoids, e.g., antioxidant, anticataract, anti-inflammatory, antiallergic effects, and they inhibit lipoxygenase activity (2-5). Some isoflavonoids also exhibit antiproliferative (6), angiogenesis-inhibiting (7), hypocholesterolemic, and triglyceride-lowering activity (8). Isoflavones, e.g., genistein, inhibit tyrosine-specific kinase activity and can modulate signal transduction from the membrane to the nucleus (9). Isoflavones such as genistein, orobol, and psi-tectorigenin decrease the expression of the c-myc gene (10) and inhibit the proliferation of colon or breast cancer cell lines (11,12). Human exposure to dietary isoflavonoids, genistein, and daidzein, is mainly through intake of soy food (13). Genistein and daidzein are thought to reduce cancer risk (14) because populations with high isoflavone exposure through soy consumption have low cancer rates (15). Furthermore, cancer incidence and severity was lowered when newborn animals were treated with only three single doses of genistein (16).

It is thought that excessive lipid peroxidation and production of reactive oxygen species such as singlet oxygen may participate in pathological processes. Singlet oxygen can be produced in the skin as a result of photosensitization reactions triggered by certain drugs or porphyrins (17,18) and

can also be generated in biological systems by dark reactions, e.g., lipid peroxidation, and by enzyme reactions such as those catalyzed by lactoperoxidase, lipoxygenase, and chloroperoxidase (19). This report examines the inhibition of microsomal lipid peroxidation and the ability of singlet oxygen quenching of some new isoflavones and isoflavans and compares the antioxidant properties of these isoflavonoids with established antioxidants such as α -tocopherol, ubiquinol-10, quercetin, rutin, and butylated hydroxytoluene.

MATERIALS

The isoflavonoids were synthesized at the Institute for Physiological Chemistry, the University of Bonn. Butylated hydroxytoluene, ubiquinone-10, rutin, and quercetin were purchased from Aldrich (Steinheim, Germany), and RRR- α -tocopherol was a gift from Henkel (Düsseldorf, Germany). Ubiquinol-10 was prepared from ubiquinone-10 by dithionite reduction. ADP, NADP⁺, glucose-6-phosphate, and glucose-6-phosphate-dehydrogenase were from Boehringer (Mannheim, Germany).

MEASUREMENT OF MICROSOMAL LIPID PEROXIDATION

Microsomes (0.5 mg protein/ml) were incubated in 0.1 M KPi buffer, pH 7.4, $t = 37^\circ\text{C}$. Lipid peroxidation was initiated with premixed ADP (1 mM)/FeCl₃ (10 μM), and NADPH regenerating system, containing NADPH⁺ (0.4 mM), glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (5 units/ml) as described previously (20). Reactions were started by addition of NADP⁺. Lipid peroxidation was assayed at the TBA test. Malondialdehyde (MDA) equivalents were estimated by the formation of thiobarbituric acid-reactive substances using $\epsilon_{535} = 156 \text{ mM}^{-1} \text{ cm}^{-1}$.

GENERATION AND QUENCHING OF SINGLET OXYGEN

Singlet oxygen was generated chemically by thermal decomposition of the endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate, NDPO₂ (21). At 37°C , 3 ml methanol/chloroform (50 : 50) were placed in a thermostatted cuvette. Reactions were started by injection of 5 mM NDPO₂. The singlet oxygen quenching constants were calculated according to Stern-Volmer plots, from $S_0/S = 1 + (k_q + k_r)[Q]I$, where S_0 and S are chemilumi-

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Lipid Peroxidation and Singlet Oxygen Quenching

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nescence (1270 nm) intensities in the absence and presence of quenchers, respectively; [Q] is the quencher concentration, and I is the lifetime of singlet oxygen.

INHIBITION OF LIPID PEROXIDATION AND SINGLET OXYGEN QUENCHING

The half-inhibition concentrations of isoflavones and isoflavans (Fig. 1) on microsomal lipid peroxidation are presented in Table 1. The most effective, isoflavone (compound 1) and isoflavan (compound 6), showed similar antioxidant activity. These compounds inhibit lipid peroxidation about 1.5-fold or 70-fold more strongly than BHT or α -tocopherol, respectively. The values for the investigated isoflavans are in a narrow range of 0.6–1.9 μ M, while for isoflavones the values were 0.6–16.1 μ M and >60 μ M. Among the isoflavones the 7,8-dihydroxyisoflavone isomers (compounds 1 and 4) exhibit stronger antioxidant activity than the 6,7-dihydroxyisoflavone isomer (compound 2). Also, 7-hydroxy-8-methyl-isoflavone (compound 3) demonstrated less potent antioxidant activity than compounds 1 and 4.

A similar structure-activity relationship was also observed for singlet oxygen quenching. 7,8-Dihydroxyisoflavones (compounds 1 and 4) were

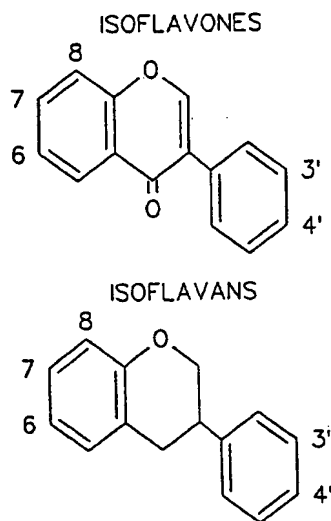


Figure 1 Structures for isoflavones and isoflavans.

Table 1 Comparison of Concentrations for Half-Inhibition of Lipid Peroxidation and Singlet Oxygen Quenching Rate Constants ($k_q + k_t$) for Different Isoflavones, Isoflavonols, and Some Antioxidants

Compound	6	7	8	3'	4'	Half-maximal inhibition of lipid peroxidation (μM)	Singlet oxygen quenching rate constants ($k_q + k_t$) $\times 10^8 \text{ M}^{-1} \text{ s}^{-1}$
Isoflavones							
1	H	OH	OH	CF ₃	H	0.6	1.6
2	OH	OH	H	CF ₃	H	3.1	<0.05
3	H	OH	CH ₃	CF ₃	H	>60	0.1
4	H	OH	OH	F	H	0.8	1.0
5	OH	OH	H	F	H	16.1	—
Isoflavonols							
6	OH	OH	H	H	OCH ₃	0.6	0.7
7	OH	OH	H	H	CH ₃	0.7	0.5
8	H	OH	OH	H	OH	1.0	0.3
9	H	OH	OH	H	OCH ₃	1.9	0.2
10	H	O-CH ₃ -O	OH	H	OCH ₃	0.8	0.3
Flavonols							
Quercetin						3	0.025
Rutin						14	0.008
Butylated hydroxytoluene							
Ubiquinol-10						0.9	0.02
Ubiquinone-10						60	0.3
α -Tocopherol						>100	0.085
β -Carotene						40	3
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more efficient singlet oxygen quenchers than 6,7-dihydroxyisoflavone (compound 2). Substitution of the methyl group at position 8 (compound 3) did not lead to a strong decrease of singlet oxygen quenching ability as in the case of antioxidant activity on microsomal lipid peroxidation.

There was no strong specific relationship for inhibition of lipid peroxidation or singlet oxygen quenching for different substituents among the tested isoflavans. However, 6,7-dihydroxyisoflavans (compounds 6 and 7) exhibit slightly higher antioxidant and singlet oxygen quenching ability than 7,8-dihydroxyisoflavans (compounds 8 and 9). On microsomal lipid peroxidation, the isoflavonoids proved to be on average two to five times more active antioxidants than quercetin (Table 1), one of the most potent antioxidants in the group of flavonoids.

Among the investigated isoflavonoids, compound 1 was the most effective singlet oxygen quencher, with an overall quenching constant of $1.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. α -Tocopherol showed similar efficiency (22). The established phenolic antioxidants such as BHT, quercetin, and rutin exhibited lower singlet oxygen quenching constants by about two orders of magnitude (Table 1).

Singlet oxygen is reactive and causes damage to DNA by preferentially reacting with guanine (23,24), can inactivate herpes simplex virus type I, and may act as a mediator of virucidal effects in photodynamic procedures (25,26). The flavonol rutin (0.35 mM) allows inactivation of platelet-associated vesicular stomatitis virus with the retention of platelet integrity upon photoinactivation by aminomethyltrimethylpsoralen and UVA (27). Rutin exhibits a relatively low singlet oxygen quenching rate constant ($8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) (Table 1). For example, the decay constants of singlet oxygen in H_2O are in the range of $3\text{--}5 \times 10^5 \text{ s}^{-1}$ (28). It appears that rutin cannot protect the virus and cells from direct singlet oxygen damage but can inhibit subsequent lipid peroxidation in membranes initiated by singlet oxygen (29,30).

Singlet oxygen also causes pathological conditions such as alterations in the respiratory epithelium (31). It is interesting to note that the singlet oxygen quenching ability of phenolic compounds correlates with their capacity to protect against benzo(a)pyrene-induced neoplasia (32). The ability of the new isoflavonoids to inhibit microsomal lipid peroxidation and to quench singlet oxygen, as shown here, and 5-lipoxygenase as shown in Ref. 5, points to a potential use of these compounds for therapy in free radical pathology.

Other biological effects of isoflavonoids that have been demonstrated may be independent of their antioxidant activities. For example, the anticarcinogenic action of isoflavonoid is correlated with binding to estrogen

receptors, antiproliferative action with regard to breast cancer cells, or inhibition of tyrosine kinases (33).

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Structure-activity relationships among isoflavonoids with regard to their antifungal properties

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In order to establish a structure-activity relationship in the class of isoflavonoids, 16 differently substituted isoflavonoids were tested against *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium oxysporum* and *Trichoderma harzianum*. The isoflavanones, 6,7-dihydroxy-4-methoxy- and 7-hydroxy-8,4'-dimethylisoflavanone showed highest antifungal activity in the case of *C. herbarum* as test fungus. The unreduced structure of the isoflavones has less inhibitory effect on the growth of the test fungi, whereas the completely reduced isoflavones, i.e. the isoflavans, showed only a very weak activity.

Isoflavonoids are products of secondary plant metabolism which possess significant antimicrobial activities (e.g. Van Etten, 1976; Wyman & Van Etten, 1978; Kaplan *et al.*, 1980). Their utilization as natural plant protectants has been discussed and may be an alternative to conventional pesticides (Mansfield & Bailey, 1984). However, in this connection positive as well as negative results have been reported (Rathmell & Smith, 1980; Weidenbömer *et al.*, 1992). For a more successful application detailed informations about structure-activity relationships are necessary to increase the knowledge about the antifungal potential of isoflavonoids. Although there are several studies which try to clarify this phenomenon (Ben-Aziz, 1967; Perrin & Cruickshank, 1969; Biswas *et al.*, 1981; Arnoldi & Merlini, 1990; Weidenbömer & Jha 1994) no general rule could be established predicting the fungicidal properties of these natural compounds up to now. In the following investigation 16 different isoflavonoids in gradually reduced states (isoflavone, isoflavanone, isoflavan) carrying various substituents at different positions have been tested against four fungi occurring on grain and protein enriched seeds (Weidenbömer & Hindorf, 1989; Weidenbömer & Kunz, 1993). As some conventional fungicides contain halogen atoms in their molecules, three halogen substituted isoflavonoids have also been included in this study.

MATERIALS AND METHODS

The four moulds *Alternaria alternata* (Fr.) Keissl., *Cladosporium herbarum* (Pers.) Link ex Gray, *Fusarium oxysporum* Schltdl. and *Trichoderma harzianum* Rifai used in this investigation belong to the group of field fungi and have been isolated from various legume seeds (Weidenbömer & Hindorf, 1989).

The effect of the isoflavonoids on growth of the test fungi was investigated in solid medium. The medium contained 30 g malt extract, 15 g agar and 3 g peptone l⁻¹ distilled water, pH 5.3. The isoflavonoids were dissolved in acetone, so that the final concentration of solvent in the medium was 1.1%. Medium (15 ml) including the particular compounds in required concentrations was transferred to Petri dishes (90 mm) and inoculated with one small piece of mycelium (1 mm diam.). The dishes were incubated at 25 °C in the dark.

Sixteen isoflavonoids (Fig. 1) were tested at two concentrations for their activity on mycelial growth of the fungi. Because phytoalexins exhibit fungicidal activity in concentrations of 10⁻⁵-10⁻³ M (Van Etten & Bateman, 1971; Smith, 1976), concentrations of 2 and 8 × 10⁻⁴ M were used in these tests. The isoflavonoids have been synthesized in the Institute of Physiological Chemistry of the University of Bonn, Germany (H. C. Jha, personal communication).

Mycelial diameters of *T. harzianum*, *A. alternata*, *F. oxysporum* and *C. herbarum* were measured after 3, 7, 7 and 14 d, respectively. Evaluation was carried out by measuring the diameter of the colonies three times at different sites. The mean value of 6 repetitions for each concentration and fungus was used for calculation. The data were evaluated by analysis of variance. Probability of single differences was calculated at the 5% level. All data are statistically significant at this level.

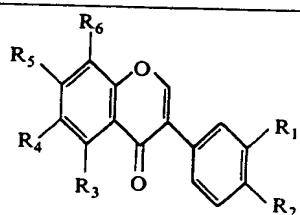
RESULTS AND DISCUSSION

This investigation shows that the unsubstituted isoflavone (1) inhibits the growth of *C. herbarum*, *F. oxysporum* and *T. harzianum* preferentially in lower concentrations of 2 × 10⁻⁴ M. Increase of the concentration to 8 × 10⁻⁴ M had an adverse effect on the activity (Table 1). In the case of *A. alternata* even

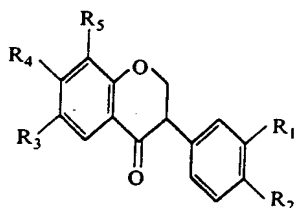
Table 1. % growth inhibition or stimulation of *A. alternata*, *C. herbarum*, *F. oxysporum* and *T. harzianum* in comparison with the corresponding control

Isoflavonoid	<i>A. alternata</i>		<i>C. herbarum</i>		<i>F. oxysporum</i>		<i>T. harzianum</i>	
	$\times 10^{-4}$							
1		+24.2	+25.8	-32.5	-13.4	-26.8	-20.5	-28.6
2		.	-3.5	.	-16.4	.	.	-11.9
3		-6.4	-9.6	.	.	-15.9	-16.6	-18.4
4		-11.5	-6.1	.	.	-29.2	-27.1	-43.9
5		-9.8	-16.1	-8.7	.	-9.2	-13.4	.
6		-9.3	-15.6	.	-8.5	-11.8	-23.8	.
7		-6.8	-25.9	+21.0	.	-7.4	-35.7	.
8		.	+8.7	.	.	-5.3	-13.9	+4.6
9		-2.4	-27.5	.	-18.2	-12.6	-45.8	.
10		-10.0	-20.5	-40.4	-52.7	.	-19.8	-1.8
11		.	.	.	-58.1	-5.3	-18.3	.
12		-5.3	-5.6	.	.	-1.9	-3.1	.
13		-20.8	-25.1	.	.	-31.2	-35.1	.
14		-12.6	-19.0	.	-12.6	.	-11.0	-1.7
15		-6.8	-20.5	-12.6	-22.9	-8.3	-38.8	.
16		+3.2	+13.5	-12.3	-	-2.7	-15.0	.

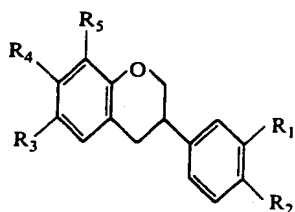
* means no significant growth inhibition, data not presented.



- 1 : $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = H$
 2 : $R_1 = R_3 = R_4 = R_6 = H, R_2 = R_5 = OH$
 3 : $R_1 = R_3 = R_4 = R_6 = H, R_2 = OCH_3, R_5 = OH$
 4 : $R_1 = R_4 = R_6 = H, R_2 = OCH_3, R_3 = R_5 = OH$
 5 : $R_1 = R_3 = R_6 = H, R_2 = OCH_3, R_4 = R_5 = OH$
 6 : $R_1 = R_3 = R_4 = H, R_2 = OCH_3, R_5 = R_6 = OH$
 7 : $R_1 = R_3 = R_4 = H, R_2 = CH_3, R_5 = R_6 = OH$
 8 : $R_1 = R_3 = R_6 = H, R_2 = R_4 = R_5 = OCOCH_3$
 9 : $R_1 = F, R_2 = R_3 = R_4 = H, R_5 = R_6 = OH$



- 10 : $R_1 = R_5 = H, R_2 = OCH_3, R_3 = R_4 = OH$
 11 : $R_1 = R_3 = H, R_2 = CH_3, R_4 = R_5 = OH$
 12 : $R_2 = R_5 = H, R_3 = R_4 = OH, R_1 = OCOCH_3$
 13 : $R_1 = R_2 = Cl, R_3 = R_5 = H, R_4 = OH$



- 14 : $R_1 = R_5 = H, R_2 = OCH_3, R_3 = R_4 = OH$
 15 : $R_1 = R_3 = H, R_2 = CH_3, R_4 = R_5 = OH$
 16 : $R_2 = R_3 = H, R_1 = F, R_4 = R_5 = OH$

Fig. 1. Isoflavonoids tested for activity on mycelial growth of fungi.

a growth promoting effect was observed in both the concentrations. Hydroxylation at 7 and 4'-positions (2) which is very common with naturally occurring isoflavonoids, did not bring any advantage; whereby the methylation of the 4'-OH group (7) indicated an upward trend as regards the activity (Table 1). Only in the case of *C. herbarum* was the 7,8-dihydroxy-4'-methoxyisoflavone (7) less active than the corresponding hydroxy-compound.

The modest activity of 7-hydroxy-4'-methoxyisoflavone (3) in the present study was also demonstrated by Van Etten (1976). Keeping the methoxy substituent intact at 4'-position, the dihydroxylated A-ring compounds (4, 5, 6) were tested for their fungicidal properties. The 5,7-dihydroxy-4'-methoxyisoflavone (Biochanin A, 4) was the most active substance against *T. harzianum* in the lower concentration (Table 1). This result is corroborated by our previous investigation (Weidenbömer *et al.*, 1990a) where this substance caused inhibition rates of 79.6 and 90.8% in the case of *Rhizoctonia solani* and *Sclerotium rolfsii*, respectively. The other two isomers (5 and 6) inhibited the growth of all test fungi to a minor extent, only (Table 1). As the 7,8-dihydroxy-4'-methoxyisoflavone (6) showed an inhibition of *F. oxysporum* up to 23.8% two more derivatives in this series, viz. 7,8-dihydroxy-4'-methyl- and 7,8-dihydroxy-3'-fluoroisoflavone (7 and 9) were included as test substances. It is of great interest that the substituents $-CH_3$ or $-F$ in ring B contribute towards making the 7,8-dihydroxyisoflavone more active against *F. oxysporum*. Isoflavones with methyl groups and halogen atoms (F, Cl, Br, I) at other positions in ring B are being tested presently to discover more active antifungal substances. Unfortunately, no isoflavones containing three substituents in ring A were available to confirm the assumption of Johnson *et al.* (1976) that they lead to a higher antifungal activity than only two or one substituent. As their acetylated derivatives are more lipophilic than the corresponding hydroxy compounds, the 6,7,4'-triacetoxyisoflavone (8) was also tested for its inhibitory activity. The inhibition value of only 13.9% against *F. oxysporum* and the growth-promoting effect in the

case of other fungi subscribe to the observation of Stössel (1985) regarding the influence of lipophilicity on the antifungal activity.

Among the tested isoflavanones, the 6,7-dihydroxy-4'-methoxy- and the 7,8-dihydroxy-4'-methyl-derivative (10 and 11) proved to be the most active ones. As in the case of isoflavones the methyl substituent in B-ring of the isoflavanone proves to be conducive to the inhibitory effect. Again the substantial activity of 7-hydroxy-3',4'-dichloroisoflavanone (13) against *F. oxysporum* shows that the halogenation of ring B could lead to more active substances (Table 1). The acetylation had a negative effect on the fungicidal activity as evidenced by 6,7-dihydroxy-3'-acetoxyisoflavanone (12). The substantial antimicrobial potential of the isoflavanones is confirmed by the result of other authors, too (Wyman & Van Etten, 1978; Fraile *et al.*, 1982), as well as by our own experiments (Weidenbömer *et al.*, 1987). It seems that two hydroxyl groups guarantee at least a moderate antifungal activity (Weidenbömer *et al.*, 1990a) because Krämer *et al.* (1984) tested four isoflavanones with other substituents which were all inactive. The introduction of another hydroxyl group (isoferreirin) even seems to enhance the fungicidal potential of isoflavanones (Adesanya *et al.*, 1986). Nevertheless, in our studies the 5,7,4'- and the 6,7,4'-trihydroxyisoflavanone did not show any considerable activity (Weidenbömer *et al.*, 1990a, b). On the other hand kievitone and its two derivatives (5-deoxykievitone) and 3'-($\gamma\gamma$ -(dimethylallyl))-kievitone were highly active isoflavanones mainly due to the presence of a dimethylallyl side chain (Wyman & Van Etten, 1978; O'Neill & Mansfield 1982; Adesanya *et al.*, 1986).

The isoflavans proved to be poor antifungal compounds in this investigation. This result is totally contrary to what we found out in the past. It is surprising that the 6,7-dihydroxy-4'-methoxyisoflavan (14) is completely ineffective against the tested fungi; although the substance caused more than 96% inhibition against *Eurotium* spp. (Weidenbömer *et al.*, 1990b). Similarly in a previous study another isoflavan (6-methoxy-7,4'-dihydroxyisoflavan) showed substantial activity against three food-contaminating fungi (Krämer *et al.*, 1984). In the present investigation the 7,8-dihydroxy-4'-methylisoflavan (15) caused only minor inhibition rates (Table 1) although the 6,7-dihydroxy-3'-methylisoflavan isomer was the most active compound with inhibition rates between 73.2 and 98.3% against five fungi of the genus *Aspergillus* (Weidenbömer *et al.*, 1990b). It is interesting to note that O'Neill & Mansfield (1982) found a negative correlation between the degree of isoflavan methylation and the fungicidal activity which was not confirmed by the results of Ingham (1977).

Although these results further contribute in clarifying structure-activity relationships of isoflavonoids more data are still necessary to establish definite rules to predict the fungicidal potential of these natural compounds.

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NEUE SYNTHESE VON ISOFLAVENEN UND VERGLEICHENDE
UNTERSUCHUNGEN IHRER ABKÖMMLINGE BEZÜGLICH IHRER
PHARMAKOLOGISCHEN EIGENSCHAFTEN

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SUMMARY

Many chromone, o-Dihydroxydesoxy-benzoine, 3-Phenylcumarine and several 3-Phenylchromene-(3) were prepared for investigation of structure-activity relationship and antioxidative characteristics. The nature of the structures were confirmed by physical chemical methods.

ZUSAMMENFASSUNG

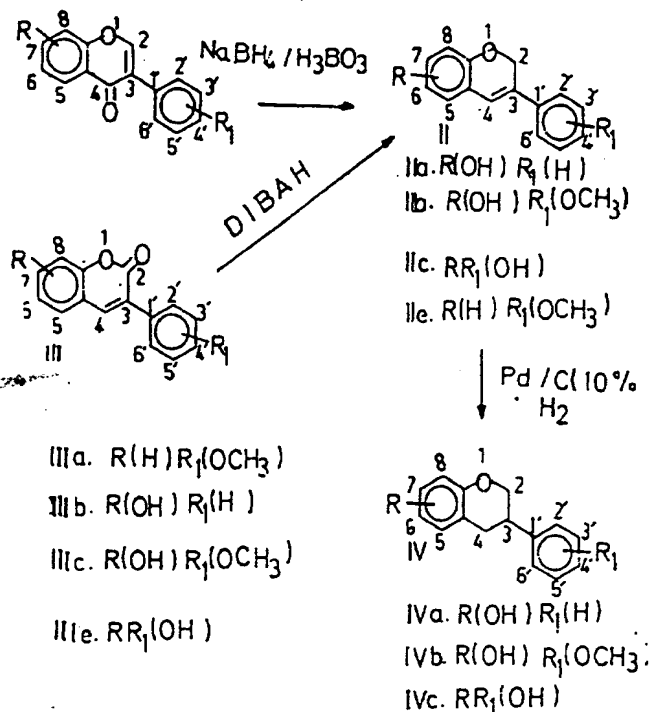
Zur Untersuchung der antioxidativen eigenschaften und der Struktur-Aktivitätsbeziehungen wurden zahlreiche Chromone, o-Dihydroxydesoxy-benzoine, 3-Phenylcumarine und einige 3-Phenylchromene-(3) hergestellt. Die Authentizität ihrer Strukturen wurde durch physikalisch-chemische Methoden sichergestellt.

3-Phenylchromene-(3) wurden zum ersten Mal aus Isoflavonen durch $\text{NaBH}_4/\text{H}_3\text{BO}_3$ erhalten. Ferner wurden auch die 3-Phenylcumarine erfolgreich mit Diisobutylaluminiumhydrid (DIBALH) zu 3-Phenylchromene-(3) geführt. Diese wurden durch die 10% ige Palladium/Aktivkohle zu Isoflavonen hydriert.

Die antioxidativen Eigenschaften aller dieser Substanzen wurden bezüglich ihrer Schutzwirkung auf Vitamin-E-freies Schweineschmalz in der Konzentration (50, 125, 250 ppm) im 72-Stunden-Test geprüft.

EINLEITUNG

Die Pflanzenphenole, die u.a. die Vorstufe der Flavonoide (Flavone und Isoflavone) darstellen, weisen pharmakologische Aktivitäten auf.^{1,2} Zur Untersuchung der antioxidativen Eigenschaften (hohe Affinität zu Sauerstoff) und der Struktur-Aktivitätsbeziehungen wurden zahlreiche Chromone, o-Dihydroxydesoxybenzoine, 3-Phenylcumarine und neue synthetische 3-Phenylchromene-(3) und deren Hydrierungsprodukte, Isoflavone, vorgenommen.



Abteilung 1 : Isoflavan-Synthese über Isoflavone bzw. 3-Phenylcumarine

EXPERIMENTELLES

Synthese der Isoflavone (3-Phenylchromene-(3))

2-Hydroxydesoxybenzoine werden nach Dyke et al³ aus Phenolen und Phenylacetonitrilen gewonnen. Die 2-Hydroxydesoxybenzoine wurden nach Bass⁴ zu Isoflavonen (I) geführt. Die folgende Versuchsbeschreibung soll als Beispiel für die allgemeine Methode zur Darstellung der Isoflavone (3-Phenylchromene-(3)) (II) dienen:

6,7-Dihydroxyisoflaven (IIa) aus 6,7-Dihydroxyisoflavin (Ia)

1.3 g (3 mmol) 6,7-Dihydroxyisoflavin wurde mit wenig absolutem Ethanol gelöst und portionsweise mit dreifacher molarer Menge an NaBH_4 versetzt und anschließend 100 mg H_3BO_3 zugegeben.

Nachdem die heftige H_2 -Entwicklung nachgelassen hatte (2-3 Stunden), wurde unter Rückfluß über Nacht gekocht.

Man säuert mit konz. HCl an und verdünnt auf das doppelte Volumen; anschließend wurde ausgeethert und die Etherphase mit Benzol: Ethanol im Rotationsverdampfer getrocknet. Umkristallisation aus Ethanol liefert 0.9 g Isoflaven (IIa).

Schmp. 152°C

$\text{C}_{15}\text{H}_{12}\text{O}_3$

Ber.: C 75.0 H 5.0

Gef.: C 75.01 H 5.01

MS: $m/e = 240$ (M^+)

6,7-Dihydroxy-4'-methoxyisoflaven (IIb)

Schmp. 142°C

 $C_{16}H_{14}O_4$ Ber. : C 71.10 H 5.50

Gef. : C 71.09 H 5.50

MS : m/e = 270 (M^+)**6,7,4'-Trihydroxyisoflaven-(3) (IIc)**

Schmp. 175°C

 $C_{15}H_{12}O_4$ Ber. : C 70.03 H 4.661

Gef. : C 70.03 H 4.66

MS : m/e = 256 (M^+)

Zur weiteren Bestätigung der Isoflavenstruktur wurden einige Milligramm in Ethanol in Gegenwart von P/C (10%) zu Isoflavan hydriert. Die hydrierten Produkte von Isoflavenen waren identisch (DC, MC)

6,7-Dihydroxyisoflavan (IVa)

Schmp. 173°C

 $C_{15}H_{14}O_3$ Ber. : C 74.38 H 5.78

Gef. : C 74.18 H 5.91

MS : m/e = 242 (M^+)**6,7-Dihydroxy-4'-methoxyisoflavan (IVb)**

Schmp. 165-166°C

Ber. : C 70.588 H 11.294

Gef. : C 70.572 H 11.31

MS : m/e = 272 (M^+)**6,7,4'-Trihydroxyisoflavan (IVc)**

Schmp. 210-211°C

 $C_{16}H_{14}O_4$ Ber. : C 69.767 H 9.762

Gef. : C 69.761 H 9.765

MS : m/e = 256 (M^+)**Synthese der 3-Phenylcumarine (III)**

Die folgende Versuchsbeschreibung soll als Beispiel für die allgemeine Methode zur Darstellung der 3-Phenylcumarine dienen :

6,7-Dihydroxy-3-Phenylcumarin (IIIb)

12.60 g (50.0 mmol) 1,3,4-Triacetoxybenzol und 8.64 g (45.0 mmol) α -Formylphenylethylsäureethylester werden unter Eiskühlung mit 50 ml 80-proz. Schwefelsäure versetzt. Die Mischung wird unter N₂-atmo-

sphäre 1 h in der Kälte gerührt und anschließend 15 min auf 80°C erhitzt. Nach dem Abkühlen wird mit 200 g Eis versetzt. Das Präzipitat wird abfiltriert und aus Ethanol umkristallisiert:

Schmp. 257-258°C (Lit. 5: 258°C)

6,7-Dihydroxy-3-(4'-methoxyphenyl) cumarin

Schmp. 256°C

$C_{16}H_{12}O_5$ Ber.: C 67.60 H 4.22

Gef.: C 67.49 H 4.32

MS: m/e = 284 (M^+)

6,7-Dihydroxy-3-(4'-hydroxyphenyl) cumarin

Schmp. 300°C

$C_{15}H_{10}O_5$ Ber.: C 66.66 H 3.70

Gef.: C 66.61 H 3.76

MS: m/e = 270 (M^+)

Neue Isoflaven-Synthese aus 3-Phenylcumarinen

Zunächst wurden die einfachen 3-Phenylcumarine (III) für die Reduktion zu den 3-Phenylchromenen (Isoflavenen) (II) in Betracht gezogen. Als Reduktionsmittel erwies sich Diisobutylaluminiumhydrid (DI-BAH) als sehr geeignet. Die Reduktion von 3-(4'-Methoxyphenyl) cumarin (IIIa) zum 4'-Methoxyisoflaven (IIc) dient als Prototyp für diese Reaktion:

4'-Methoxyisoflaven (IIc) aus 3-(4'-Methoxyphenyl) cumarin (IIa)

Zu einer Lösung von 150.0 g (0.595 mmol) 3-(4-Methoxyphenyl) cumarin in 10 ml wasserfreiem THF wird bei -70 bis -80°C (Aceton/Trockeneis-Kühlung) unter N_2 0.3 ml (70 % ige Lösung; 1.5 mmol) DI-BAH langsam getropft. Das Gemisch wird eine Stunde bei Raumtemperatur gerührt und vorsichtig mit 2N H_2SO_4 angesäuert. Nach weiteren 30 min Rühren wird die Mischung mit 10 ml Eiswasser versetzt und mit Ether extrahiert (2x20 ml). Der Extrakt wird mit Wasser gewaschen und über Na_2SO_4 getrocknet. Nach Eindampfen in Vakuum und Umkristallisation aus Ethanol erhält man 115 mg Produkt.

Schmp. 152°C

$C_{16}H_{14}O_2$ Ber.: C 67.22 H 5.88

Gef.: C 67.13 H 5.82

MS: m/e = 238 (M^+)

Zur weiteren Bestätigung der Isoflaven-Struktur wurden einige Milligramm in Ethanol in Gegenwart von Pd/C (10 %) hydriert. Das hydrierte Produkt war identisch (DC, MS und Elementaranalyse) mit dem 4'-Methoxyflavan, das aus 4'-Methoxyisoflaven zum Vergleich hergestellt wurde.

Schmp. 109°C

$C_{16}H_{16}O_2$ Ber.: C 80.00 H 6.66

Gef.: C 79.89 H 6.62

MS: m/e = 240 (M^+)

3',4'-Dimethoxyisoflaven aus 3-(3', 4'-Dimethoxyphenyl) cumarin (IIIe)

0.5 g Cumarin werden, wie oben beschrieben, durch Reduktion mit DIBAH zum 3'-4'-Dimethoxyisoflaven überführt : 0.37 g.

Schmp. 139°C

$C_{17}H_{16}O_3$ Ber. : C 76.11 H 5.97

Gef. : C 76.23 H 6.01

MS : m/e = 268 (M^+)

Dieses Isoflaven wurde in Ethanol mit Pd/C (10 %) zum 3',4'-Dimethoxy-isoflavan katalytisch hydriert.

Schmp. 83°C

$C_{17}H_{18}O_3$ Ber. : C 75.55 H 6.66

Gef. : C 75.41 H 6.41

MS : m/e = 270 (M^+)

Methodik Des Antioxidantestes

2.0 g Stripped Lard werden in 5 ml-Glasfläschchen eingewogen. Von den Testsubstanzen wird eine Stammlösung in Chloroform/Methanol (1:1, V/V) unbekannter Konzentration bereitet. Man wiegt jeweils 50 μ l in dieser Stammlösung auf einer Cahn-Mikrowaage ab und ermittelt so die in 50 μ l enthaltene Mikrogrammenge. Aus drei Wägungen pro Testsubstanz wird der Mittelwert berechnet.

In allen durchgeführten Vorversuchen konnten die folgenden Substanzkonzentrationen für alle Antioxidantests als geeignet betrachtet werden:

Testsubstanzkonzentration / 2 g Lard :

100 μ g/2 Lard = 50 ppm

250 μ g/2 g Lard = 125 ppm

500 μ g/2 Lard = 250 ppm

In einigen Fällen, je nach dem Umfang des Oxidantestes, wurden Konzentrationen niedriger als 50 ppm oder höher als 250 ppm getestet. Mit der Eppendorfpipette wird die gewünschte Konzentration der Substanz in 2 g Lard durch Hinzufügen des berechneten Volumens der Testsubstanzlösung in die Feutfläschchen erreicht.

Damit die Substanzlösung sich möglichst gut mit Fett mischt, werden die Fläschchen verschlossen und im Wasserbad bei ca. 35°C unter vorsichtigem Drehen erwärmt.

Anschließend werden sie geöffnet und 72 h im Ofen bei 60°C inkubiert. Aus diesen Proben werden genau 0.5 g Fett in 300 ml-Erlenmeyerkolben abpipettiert. Dazu gibt man 10 ml Chloroform (um das Fett in Lösung zu bringen), 15 ml Essigsäure und 1 g Kaliumiodid. Der ganze Kolbeninhalt wird 30 min auf dem Wasserbad (100°C) zum Sieden erhitzt. Anschließend kühlt man die Erlenmeyerkolben im Eisbad ab, verdünnt deren Inhalt mit 75 ml dest. Wasser und titriert ihn gegen 0.001 N Natriumthiosulfat-Lösung, wobei 1-2 Tropfen 1 % iger Stärkelösung als Indikator dienen. Aus dem ml-Verbrauch der Thiosulfatlösung der Proben läßt sich die Schutzwirkung in Prozent berechnen.

Zusammen mit jedem Testansatz wird eine Kontrolle (K) (nur Lard) unter gleichen Bedingungen inkubiert und ein Leerwert (Le) (nicht inkubiertes Fett der gleichen charge) bestimmt. Beide Werte gehen in die spätere Rechnung ein.

Berechnung der Schutzwirkung

K = ml - Verbrauch 0.001 N Natriumthiosulfatlösung der Kontrolle

Le = ml - Verbrauch 0.001 N Natriumthiosulfatlösung des Leerwertes

Pr = ml - Verbrauch Thiosulfatlösung der Proben

$$\% \text{Oxidation} = \frac{100 \times (Pr - Le)}{(K - Le)} \quad \% \text{Schutz} = 100 - \frac{100 \times (Pr - Le)}{(K - Le)}$$

ERBEBNISSE DER ANTIOXIDATIVEN WIRKUNG

Die Ergebnisse sind den folgenden Tabellen zu entnehmen.

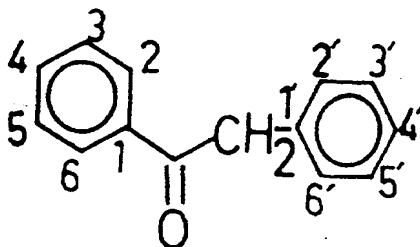


Tabelle: I - Antioxidative Wirkung von Desoxybenzoinen

Nr.	Testsubstanzen : Desoxybenzoine	% Schutzwirkung bei		
		50 ppm	125 ppm	250 ppm
1.	2,4-Dihydroxy-5-methoxyphenyl-benzylketon	5.6	13.5	19.5
2.	2,4,5-Trihydroxyphenyl-3',4'-dimethoxy-benzylketon	99.2	99.4	99.4
3.	2,4,5-Trihydroxyphenyl-2'-methoxy-benzylketon	99.6	99.9	99.7
4.	2,4,5-Trihydroxyphenyl-4'-propyloxy-benzylketon	99.6	100.0	100.0
5.	2,4,5-Trihydroxyphenyl-4'-methoxy-benzylketon	100.0	99.4	100.0
6.	2,4,5-Trihydroxyphenyl-2'-butyloxybenzylketon	99.9	100.0	100.0
7.	2,4,5-Trihydroxyphenyl-4'-hydroxy-benzylketon	97.2	99.9	100.0
8.	2,4,5-Trihydroxyphenylbenzylketon	96.9	99.7	99.3
9.	2-Hydroxy-5-methoxyphenyl-4'-methoxy-benzylketon	-	-	-
10.	2-Hydroxy-4-methoxyphenyl-4'-methoxy-benzylketon	49.7	52.3	54.1

Tabelle : I

Nr.	Testsubstanzen : Desoxybenzoine	% Schutzwirkung bei		
		50 ppm	125 ppm	250 ppm
11.	2,4-Dihydroxy-3',4'-dimethoxybenzylketon	-	-	-
12.	2-Hydroxy-4-methoxyphenyl-4'-methoxybenzylketon	-	-	-
13.	2,4,6-Trihydroxyphenyl-3',4',5'-trimethoxybenzylketon	11.6	9.7	5.3
14.	2,4,6-Trihydroxyphenyl-4'-hydroxybenzylketon	5.5	12.8	41.8
15.	2,4,6-Trihydroxyphenyl-3',4'-dimethoxybenzylketon	-	-	-
16.	2-Hydroxy-3,4-dimethoxyphenylbenzylketon	-	-	-
17.	2-Hydroxy-3,4-dimethoxyphenyl-4'-hydroxybenzylketon	-	-	-
18.	2-Hydroxy-4,5-dimethoxyphenyl-3',4'-dimethoxybenzylketon	3.3	4.1	6.9
19.	2,3,4-Trihydroxyphenyl-4'-methoxybenzylketon	77.4	94.8	99.5
20.	2,4,5-Triacetoxyphenyl-4'-methoxybenzylketon	40.0	61.3	53.5
21.	2-Hydroxy-4,5-dimethoxyphenyl-3',4'-dimethoxybenzylketon	98.6	100.0	100.0
22.	2-Hydroxy-4,5-dimethoxyphenyl-4'-methoxybenzylketon	-	-	-
23.	2-Hydroxy-4,5-dimethoxyphenyl-4'-nitrobenzylketon	53.8	26.9	50.3
24.	2-Hydroxy-4,5-dimethoxyphenylbenzylketon	-	-	29.5
25.	2-Hydroxy-4,5-dimethoxyphenyl-4'-hydroxybenzylketon	-	-	-
26.	2,5-Dihydroxy-4-methoxyphenyl-4'-methoxybenzylketon	32.0	79.5	95.3
27.	2,5-Dihydroxy-4-methoxyphenyl-3',4'-dimethoxybenzylketon	43.4	71.2	92.3
28.	2,5-Dihydroxy-4-methoxyphenylbenzylketon	35.5	83.8	92.2
29.	2,5-Dihydroxy-4-methoxyphenyl-4'-hydroxybenzylketon	20.8	54.0	90.8
30.	2,4-Dihydroxy-5-methoxyphenyl-4'-methoxybenzylketon	-	-	-

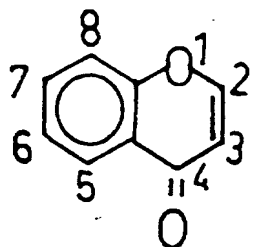


Tabelle : II- Chromone

Nr.	Testsubstanzen : Chromone	% Schutzwirkung bei		
		50 ppm	125 ppm	250 ppm
1.	6-Hydroxy-7-methoxy-3-methyl-	-	31	45.5
2.	7-Hydroxy-6-methoxy-3-methyl-	40	45	51.9
3.	6,7-Dihydroxy-3-propyl-	18.8	45.6	89
4.	6,7-Dihydroxy-3-isopropyl-	-	10.6	34.2
5.	6,7-Dihydroxy-3-methyl-	-	50.6	83.2
6.	6,7-Dihydroxy-3-ethyl-	-	66.2	88

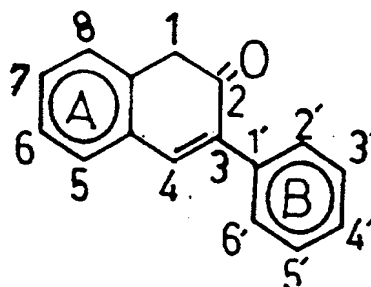


Tabelle : III- 3-Phenylcoumarine

Nr.	Testsubstanzen : 3-Phenylcoumarine	% Schutzwirkung bei		
		50 ppm	125 ppm	250 ppm
1.	6,7-Dihydroxy-4'-methoxy-	99	99.2	99.9
2.	6,7-Dihydroxy-	99.1	99.4	100
3.	6,7-4'-Trihydroxy-	99.6	99.8	100

Tabelle : IV- 3-Phenylchromene-(3)

Nr.	Testsubstanzen : Isoflaven-(3)	% Schutzwirkung bei		
		50 ppm	125 ppm	250 ppm
1.	6,7-Dihydroxy	99.6	100	100
2.	6,7-Dihydroxy-4'-methoxy-	85	99	100
3.	6,7-4'-Trihydroxy-	99	99.9	100

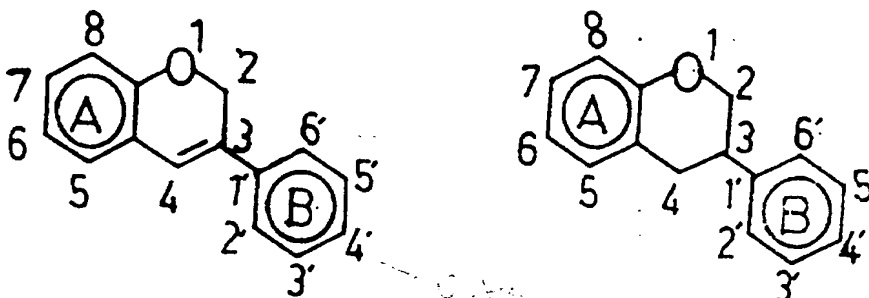


Tabelle : V- Isoflavane

Nr.	Testsubstanzen : Isoflaven-(3)	% Schutzwirkung bei		
		50 ppm	125 ppm	250 ppm
1.	6,7-Dihydroxy-4'-methoxy-	88.2	98.2	100.0
2.	6-Hydroxy-7-methoxy-	94.2	94.4	94.4
3.	6,7,4'-Trihydroxy	42.0	97.1	99.4
4.	6,7-Dihydroxy-	95.6	99.3	99.7
5.	6,4'-Dimethoxy-7-hydroxy-	-	79.4	89.8
6.	6,7-Dimethoxy-4'-hydroxy-	-	-	-
7.	7-Hydroxy-6-methoxy-	64.7	83.3	60.4
8.	7,4'-Dihydroxy-6-methoxy-	53.6	74.0	90.6
9.	7,4'-Dimethoxy-6-hydroxy-	92.8	95.2	94.1

DISKUSSION

Es konnten mehrere Isoflavene aus den entsprechenden Isoflavonen gewonnen werden. In diesem Zusammenhang mußten einige Isoflavone zuerst synthetisiert werden (Abb. I). Die herkömmlichen Methoden^{6,7} wurden durch neuere ersetzt^{4,8,9}. Die Isoflavene wurden durch katalytische Hydrierung zu Isoflavanen geführt.

Eine weitere Isoflavan-Synthese wurde über die entsprechenden 3-Phenylchromene erreicht. Das Reagenz Diisobutylaluminiumhydrid (DIBALH) konnte aus 3-Phenylcumarinen die 3-Phenylchromene Isoflavene-(3) bilden, die anschließend leicht katalytisch (10 % ige Pd/C; H₂) hydriert werden konnten (Abb. I).

Nachdem die Substanzen synthetisiert und durch Elementaranalyse und spektroskopische Methoden auf ihre Authentizität hin untersucht worden waren, wurden sie für die antioxidativen Studien eingesetzt.

Der Antioxidans-Test ergibt, daß Chromone, 3-Phenylchromene, 3-Phenylcumarine und Isoflavane mit einer o-Dihydroxy-Gruppe im A-Ring eine hohe Wirkung besitzen. Bei den Chromonen führt eine Verlängerung der C-Kette in Position 3 zu einer kontinuierlichen Aktivitätserhöhung. Eine Verzweigung dieser Kette verursacht jedoch eine negative Wirkung. Die Einführung einer zusätzlichen OH-Gruppe im B-Ring erhöht die Aktivität. Diese Wirkung läßt sich dadurch erklären, daß die Phenole Radikal-"Scavengers" sind.

Die o-Dihydroxygruppe wird außerdem leicht zum Chinon oxidiert, wodurch Antioxidation vermieden wird. Auch bei o-Hydroxygruppen in 6,7-Position im A-Ring des 3-Phenylcumarins ist eine gute Wirkung zu beobachten. Eine höhere Aktivität der Isoflavene und Isoflavane entsteht, weil durch Reduktion des C-Ringes die Lipophilität erhöht wird, sich eine Annäherung an die Vitamin-E-Chromonstruktur ergibt.

Bei den 2-Hydroxydesoxybenzoinen wird durch die Einführung weiterer OH-Gruppen in o- und p-Stellung eine o- bzw. p-Chinoidbildung begünstigt und dadurch das gebildete freie Radikal zerstört.

Die m-Dihydroxy-Verbindungen zeigen keine Aktivität, wie von der schlechten Oxidierbarkeit der Resorcin- oder Phloroglucinstruktur zu erwarten ist.

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NEW ISOFLAVONOIDS AS INHIBITORS OF PORCINE 5-LIPOXYGENASE

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Abstract—The inhibitory activity of new isoflavonoids on 5-lipoxygenase of porcine leukocytes was investigated. Isoflavans (I) proved to be stronger inhibitors than isoflavones (II). The isoflavans containing *ortho*-hydroxy groups in ring A showed the lowest K_i values (0.8–50 μM). In comparison, isoflavans with *meta*-dihydroxy groups exhibited K_i values higher than 150 μM . The effect of commercial antioxidants was tested also on porcine 5-lipoxygenase. Butylated hydroxyanisole (K_i : 25 μM) and butylated hydroxytoluene (K_i : 55 μM) revealed moderate inhibitory activity, whereas L-ascorbic acid, L-ascorbyl palmitate, *dl*- α -tocopherol and *n*-propyl gallate showed weak inhibitory activities (K_i : 100–260 μM).

Isoflavonoids represent an important subclass of the flavonoids. The structure of isoflavonoids is based on a 3-phenylchroman skeleton, which biogenetically is derived by aryl migration from the 2-phenylchroman skeleton of the flavonoids [1]. While flavonoids are ubiquitous compounds, the isoflavonoids show a limited distribution in the plant kingdom. They are found mainly in the subfamily *Papilionoidae* of the Leguminosae (for review see Ref. 2).

Isoflavonoids are known to exhibit various biological properties, e.g. the insecticidal activity of rotenoids [2] and isoflavans [3], the anti-microbial, especially the anti-fungal, activity of the phytoalexins (pterocarpans, isoflavans and some isoflavones [2, 4]), an hypocholesterolemic effect and a triglyceride-lowering activity [5–7]. The isoflavonoids have many properties in common with the flavonoids, e.g. anti-cataract [8, 9] anti-inflammatory and anti-allergic activity [10–14]. Some biological activities are explained by special biochemical mechanisms. Thus, the anti-inflammatory and anti-allergic activity of flavonoids is in part due to inhibition of the enzymes involved in the arachidonic cascade [10–14].

One of the enzymes of the arachidonic acid cascade, the 5-lipoxygenase (5-LOX[†]) is the first enzyme in the biosynthetic pathway leading to LT. LTs are potent mediators, involved in immunoregulation and in various diseases, including inflammation, asthma and diverse allergic reactions. In neutrophils, stimulated with the Ionophore A 23187, the main products of arachidonic acid are 5-HETE and LTB₄ [15]. The same pattern of 5-LOX products is obtained with neutrophils derived from various species stimulated under the same conditions [16]. Previously, the HETEs were considered to be predominantly inactivation products of HPETEs

without biological importance. New investigations have shown that HETEs modulate basic biological functions such as enzyme regulation, hormone secretion, ion transport and immune mechanisms. They are involved in pathological processes including various inflammatory diseases, arteriosclerosis and ischemia (for review see Ref. 17).

Due to the participation of LT and HETEs in various diseases, we evaluate in this study the inhibitory effect of different new synthetic isoflavonoids on porcine 5-LOX *in vitro*. We also compare the effect of isoflavonoids on porcine 5-LOX with that of commercial food antioxidants.

MATERIALS AND METHODS

Materials

All isoflavonoids have been synthesized at the Institute for Physiological Chemistry of the University of Bonn [18]. The antioxidants *dl*- α -tocopherol, BHT, BHA and L-ascorbic acid were purchased from Merck (Darmstadt, Germany), *n*-propyl gallate from Sigma (Munich, Germany) and ascorbyl palmitate from Serva Feinbiochemica (Heidelberg, Germany). Dextran T-500 for cell sedimentation was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Arachidonic acid (Merck) was purified by silicic acid column chromatography prior to use. The Ionophore A 23187 and PGB₂, which served as internal standard, were obtained from Sigma. ETYA was supplied by Hoffmann-La-Roche (Basle, Switzerland). All salts, organic solvents, thin layer silicic acid plates and Trypan blue were obtained from Merck. All chemicals used were of reagent grade. The solvents for HPLC were dried, distilled and filtered.

5-LOX assay

Preparation of leukocyte suspension. Porcine peripheral blood leukocytes were prepared according to the method of Kuhl *et al.* [10]. Porcine blood (1.5 L) was decoagulated with 100 mL Hank's buffer solution containing 3.8% sodium citrate, 6 U of heparin/mL of blood and passed through a Dextran

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† Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; LOX, lipoxygenase; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; PG, prostaglandin; ETYA, 5,8,11,14-eicosatetraynoic acid.

(6%) gradient. After sedimentation at 4° for 60 min, the supernatant was centrifuged at 500 g for 12 min. The pellet was resuspended in Hank's buffer containing 0.38% sodium citrate. The centrifugation was repeated. Residual erythrocytes were lysed after 5 min incubation at 25° with Tris buffer (17 mM, pH 7.2) containing 0.17% ammonium chloride. After centrifugation at 400 g for 6 min and resuspension of the cells in Hank's buffer containing 0.38% sodium citrate, the solution was centrifuged again at 400 g for 6 min. The cell pellet was resuspended in phosphate-buffered (25 mM, pH 7.4) isotonic saline at 4×10^7 cells/mL. The viability of the cells (higher than 90%) was checked by Trypan blue exclusion.

Incubation conditions for porcine leukocytes. Leukocyte suspension (10 mL) was preincubated for 3 min at room temperature in the presence of different concentrations (0.5–200 μ M) of isoflavonoids or usual food antioxidants dissolved in ethanol or dimethyl sulfoxide. An equivalent suspension with solvent (ethanol or dimethyl sulfoxide) but lacking test substance served as control. The solvent content did not exceed 1%, to avoid an influence on 5-LOX activity [19]. During the assay for 5-LOX activity, nearly complete inhibition of 12-LOX is achieved by addition of 10 μ M ETYA [11] to the reaction mixture, as the latter enzyme is the predominant LOX of porcine leukocytes [20].

The 5-LOX reaction was started by adding the following substances to the leukocyte suspension: CaCl_2 (2 mM), Ionophore A 23187 (10 μ M), ETYA (10 μ M) and the substrate arachidonic acid (100 μ M). After incubating the cells for 5 min at 37°, the enzymatic reactions were stopped by adding 1.5 mL of formic acid (1%). After addition of PGB_2 (2 μ g) as an internal standard, the samples were extracted immediately with chloroform/methanol (1:1, v/v; 2×15 mL), evaporated and stored under nitrogen at -18° prior to HPLC analysis.

HPLC analysis. Analytical HPLC was performed according to Kuhl *et al.* [10] with slight modifications. A prepacked column (Hibar RT, 250×4 mm, Lichrosorb 60, 7 μ m, Merck) and a precolumn (RCSS Silica T 61031) from Waters, Millipore (Eschborn, Germany) were employed (instrument: S 101, Siemens; pump: DMR-AE-10.4, Orlita; Injectorsystem: U6K, Waters).

The compounds were eluted using first *n*-hexane/2-propanol/methanol/acetic acid (972/18/9/1 by vol.) containing 0.06% water. After 9.5 min the gradient elution was started. The 2-propanol content was raised during a linear gradient up to 3 vol.% in 12 min (972/30/9/1). The flow rate was 3.5 mL/min at 22°. The elution was monitored spectrophotometrically at 235 nm (0–9.5 min) and at 280 nm (9.5–24 min). 5-HETE and LTB_4 were quantified by comparing their peak areas with that of PGB_2 (internal standard). The extinction coefficients used for 5-HETE, LTB_4 and PGB_2 were $\epsilon = 30,500$, 39,500 and 26,800 $\text{L mol}^{-1} \text{mm}^{-1}$, respectively.

RESULTS

Inhibition of porcine 5-LOX by isoflavonoids

Arachidonic acid incubated with porcine leuko-

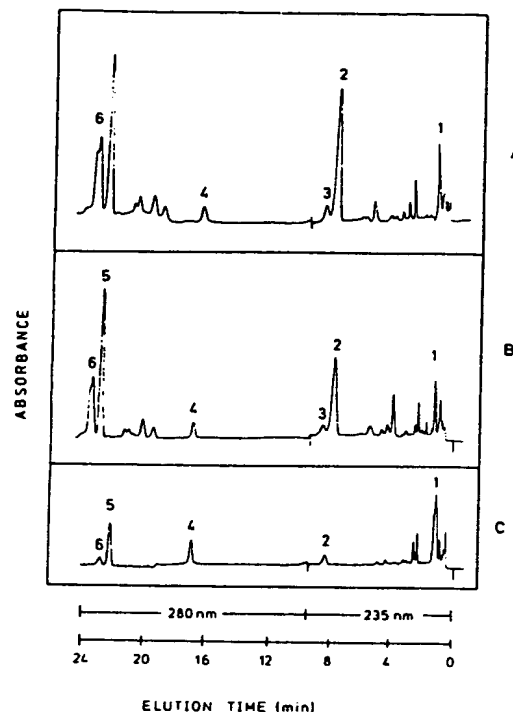
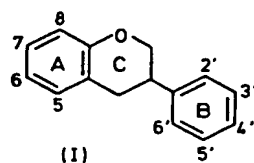


Fig. 1. Inhibition of porcine 5-LOX by 7,8-dihydroxy-4'-methoxyisoflavan (6). HPLC chromatograms of the products formed during a 5-min incubation of porcine peripheral blood leukocytes ($10 \text{ mL}/4 \times 10^7$ cells/mL) with arachidonic acid (100 μ M), CaCl_2 (2 mM), Ionophore A 23187 (10 μ M) and ETYA (10 μ M). (A) Control (without test substance), (B) with 10 μ M 7,8-dihydroxy-4'-methoxyisoflavan (6), (C) with 50 μ M 7,8-dihydroxy-4'-methoxyisoflavan (6). Signal attenuation was three times higher at 235 than at 280 nm. Peaks: 1, arachidonic acid; 2, 5-HETE; 3, 5-HPETE; 4, PGB_2 ; 5, LTB_4 ; 6, stereoisomers of LTB_4 .

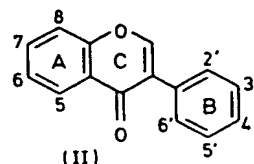
cytes in the presence of Ionophore A 23187, CaCl_2 and ETYA is converted predominantly to 5-HETE and LTB_4 . Figure 1A shows a typical HPLC chromatogram of an incubation performed under the conditions described in Materials and Methods. The effect of various concentrations of 7,8-dihydroxy-4'-methoxyisoflavan (6) on 5-LOX-activity is shown in Fig. 1B and C. The formation of the 5-LOX products 5-HETE and LTB_4 was suppressed by 7,8-dihydroxy-4'-methoxyisoflavan (6) in a dose-dependent manner. The inhibition of 5-LOX by isoflavonoids is expressed as the percentage of activity related to the control value measured without inhibitor. Plotting of $1/(\% \text{ activity})$ vs $[I]$ was carried out to evaluate the inhibition constant K_i . The substitution patterns of isoflavans (Table 1) and isoflavones (Table 2) are listed with their corresponding K_i values. K_i values of most isoflavonoids tested ranged from 0.1–100 μ M.

Structure-activity studies showed that the isoflavans inhibited porcine 5-LOX more effectively than the corresponding isoflavones [see compounds (2) and (3), (13) and (14), (18) and (17)]. Looking

Table 1. K_i values of porcine 5-LOX inhibition by various isoflavans

Compound	Substitution						K_i (μ M)
	5	6	7	8	3'	4'	
(1)	H	OH	OH	H	H	OH	37
(2)	H	H	OH	OH	H	OH	0.8
(4)	H	O-Ace	O-Ace	H	H	OCH ₃	15.5
(5)	H	OH	OH	H	H	OCH ₃	2.5
(6)	H	H	OH	OH	H	OCH ₃	21
(7)	H	OH	OH	H	H	CH ₃	53
(8)	H	H	OH	OH	H	CH ₃	7.5
(9)	OH	H	OH	H	H	CH ₃	218
(10)	H	OH	OH	H	OCH ₃	OCH ₃	7
(11)	H	H	OH	OH	OCH ₃	OCH ₃	24
(12)	H	H	OH	CH ₃	OCH ₃	OCH ₃	70
(13)	H	OH	OH	H	O-CH ₂ -O		28
(18)	H	O-CH ₂ -O		H	H	OCH ₃	0.6
(22)	H	H	OH	OH	H	Cl	9.5
(23)	H	H	OH	CH ₃	H	Cl	36
(25)*	OH	H	OH	H	H	OH	168

* See Ref. 11.

Table 2. K_i values of porcine 5-LOX inhibition by various isoflavones

Compound	Substitution						K_i (μ M)
	5	6	7	8	3'	4'	
(3)	H	H	OH	OH	H	OH	19.5
(14)	H	OH	OH	H	O-CH ₂ -O		103
(15)	H	H	OH	OH	O-CH ₂ -O		23.5
(16)	H	O-CH ₂ -O		H	OCH ₃	OCH ₃	250
(17)	H	O-CH ₂ -O		H	H	OCH ₃	60
(19)	H	OH	OH	H	F	H	16.5
(20)	H	H	OH	OH	F	H	21
(21)	H	H	OH	OH	CF ₃	H	16
(24)	H	H	OH	OH	H	NO ₂	91

for an influence of the substituents in ring B, it is obvious that there is no specific relation to 5-LOX inhibition. Among the tested isoflavans and isoflavones neither size, position nor different charge of the substituents was decisive for inhibition strength (see compounds (3), (6), (8), (11), (20), (21), (22)).

Comparing the effects of 6,7-dihydroxyisoflavonoids and of the 7,8-dihydroxyisoflavonoid

isomers on porcine 5-LOX, there was no structure-activity relationship [see compounds (1) and (2), (5) and (6), (10) and (11), (19) and (20)]. In contrast, structure-activity relationships are obvious comparing *ortho*-hydroxy- and *meta*-hydroxy-substituted compounds in ring A. Among the 4'-methylisoflavans, the *ortho*-hydroxy isoflavans (7) and (8) are significantly stronger inhibitors of porcine 5-

Table 3. Inhibition of porcine 5-LOX by commercial food antioxidants (K_i values)

Compound	K_i (μM)
BHA	25
BHT	55
<i>n</i> -Propyl gallate	124
<i>dl</i> - α -Tocopherol	259
L-Ascorbic acid	100
L-Ascorbyl palmitate	239

LOX than the respective *meta*-hydroxy isoflavan (9). A similar result was obtained in the corresponding series of 4'-hydroxyisoflavans [see compounds (1), (2) and (25)]. Comparing 7,8-dihydroxyisoflavans with the 7-hydroxy-8-methylisoflavans, the *ortho*-hydroxy substituted compounds are as expected more effective 5-LOX inhibitors [see compounds (11) and (12), (22) and (23)]. Surprisingly some isoflavans, which lack free *ortho*-dihydroxy-substituents in ring A show a marked 5-LOX inhibition. These compounds are 6,7-methylenedioxy-4'-methoxyisoflavan [(18), K_i : 0.6 μM] and 6,7-diacetyl-4'-methoxyisoflavan [(4), K_i : 15.5 μM].

Inhibition of porcine 5-LOX by food antioxidants

In Table 3 the K_i values for inhibition of porcine 5-LOX by commercially available food antioxidants are summarized. Whereas BHA (K_i : 25 μM) and BHT (K_i : 55 μM) are moderate inhibitors, L-ascorbic acid, L-ascorbyl palmitate, α -tocopherol and *n*-propyl gallate are less effective inhibitors (K_i : 100–260 μM) than most isoflavans.

DISCUSSION

Inhibition of porcine 5-LOX by isoflavonoids and food antioxidants

The different isoflavonoids tested in this study inhibited the porcine polymorphonuclear leukocytes 5-LOX in a concentration range of 0.1–100 μM (K_i and IC_{50} values). The same concentration range was obtained with most flavonoids containing also hydroxy- and methoxy-substituents [21–25]. The

absolute inhibition values found for various compounds in the literature, however, differ depending on the type of LOX, the enzyme source, method of enzyme isolation and the assay conditions. This fact is illustrated in Table 4 for quercetin, which may be designated as reference flavonoid. Therefore comparison of different studies should be undertaken with certain reservations.

Other compounds tested as inhibitors of porcine 5-LOX are: (*E/Z*)-Ajoene (IC_{50} : 1.6 μM), a garlic constituent [30]; nordihydroguaiaretic acid (IC_{50} : 1.5 μM); caffeic acid (IC_{50} : 46 μM); *p*-coumaric acid (IC_{50} : 2.5 μM) and wedelolactone (IC_{50} : 2.5 μM), a coumestane derivative [19]. These compounds inhibited the 5-LOX also to the same extent as the isoflavonoids tested in this study.

The structure-activity relationship of the isoflavonoids concerning 5-LOX-inhibition (Tables 1 and 2) revealed the following conclusions: (i) isoflavans were found to be more effective inhibitors than their corresponding isoflavones. An explanation may be the change in conformation of ring C after hydration of the isoflavones to isoflavans and the interruption of the fully conjugated system. (ii) *ortho*-Dihydroxy-substituted isoflavans (1), (2), (7) and (8) inhibited the 5-LOX at lower concentrations than the respective *meta*-dihydroxy-substituted isoflavans (9) and (33). The 7,8-dihydroxyisoflavans also proved to be stronger 5-LOX inhibitors than the 7-hydroxy-8-methyl-isoflavans (12) and (23).

Other authors [31] found that phenolic *ortho*-dihydroxy-compounds, including caffeic acid and the flavonoids taxifolin (3,5,7,3',4'-pentahydroxyflavanon), luteolin (5,7,3',4'-tetrahydroxyflavon) and quercetin (3,5,7,3',4'-pentahydroxyflavon), clearly had strong radical-scavenging activities, whereas monohydroxylated and *para*-dihydroxylated compounds proved to be only moderate to weak radical scavengers. Compounds lacking a free hydroxy-group in the molecule scarcely influenced radical scavenging. Some isoflavonoids examined in our study may also trap radicals. During this reaction isoflavonoids are oxidized to *ortho*- and/or *para*-benzoquinones as shown here for 6,7- and 7,8-dihydroxyisoflavans in Fig 2a and b.

Oxygen radicals, which are probably involved in inflammatory and cancerogenic processes, are generated as by-products during arachidonic acid metabolism via the cyclo-oxygenase and LOX

Table 4. Inhibition (IC_{50} values) of various LOXs by quercetin

Enzyme source		IC_{50} (μM)	Literature
5-LOX	RBL-1	0.2	[26]
5-LOX	RBL-1	2.1	[22]
5-LOX	RBL-1	0.1–1	[23]
5-LOX	RBL-1	>1	[21]
5-LOX	Porcine leukocytes	0.8	[11]
5-LOX	Human leukocytes	≈ 125	[27]
12-LOX	Human thrombocytes	4–5	[26]
13-LOX	Soybeans	2–3	[28]
15-LOX	Soybeans	>10	[29]

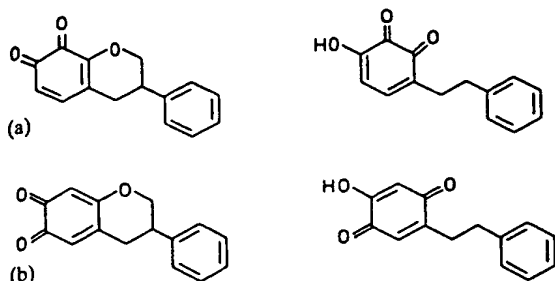


Fig. 2. (a) *ortho*-Benzoquinones of 7,8-dihydroxyisoflavan. (b) *ortho*- and *para*-Benzoquinones of 6,7-dihydroxyisoflavan.

pathway. It is known that fatty acid hydroperoxides formed in LOX reactions are necessary for LOX activity [32–35]. Therefore, trapping of these radicals by isoflavonoids may reduce the 5-LOX activity. But not all compounds with radical-scavenging properties are effective antioxidants.

LOXs are non-heme iron-containing enzymes [36, 37] existing in two different forms: a Fe^{3+} (ferric) and a Fe^{2+} (ferrous) form [38, 39]. Kemal *et al.* [34] showed that catechols reduced the catalytically active ferric of soybean LOX to the inactive ferrous form. The reduction of the Fe^{3+} LOX to the inactive Fe^{2+} form also may be performed by *ortho*-dihydroxyisoflavonoids, according to the redox cycle for LOX activation and inactivation proposed by Hatzelmann *et al.* [35]. Isoflavonoids may act as the H-atom donor and/or the radical scavenger in this cycle.

6,7-Diacetyl-4'-methoxyisoflavan (4) and 6,7-methylenedioxy-4'-methoxyisoflavan (18) which both lack the free hydroxy- group showed a strong inhibition of 5-LOX. The diacetyl- and the methylenedioxy- groups may be split off by cellular hydrolases to yield free hydroxy- groups.

The antioxidative activity of the isoflavonoids studied was examined in our laboratories [6, 7, 40]. Isoflavans, especially those containing *ortho*-dihydroxy- groups in ring A, inhibited strongly the auto-oxidation of vitamin E-free lard (this is for the most part in accordance with the structure-relationship found for 5-LOX inhibition). 6,7-Dihydroxyisoflavonoids from fermented soy oil were 10–20 times stronger antioxidants than vitamin E [41–43].

Comparing the effect of *dl*- α -tocopherol and the *ortho*-dihydroxyisoflavans on porcine 5-LOX, the isoflavonoids proved to be on average 150 times more active than the tocopherol. K_i values for *n*-propyl gallate, L-ascorbic acid and ascorbyl palmitate were also higher than those for the isoflavans. The inhibitory potency of BHA and BHT is comparable with the inhibition caused by isoflavonoids. However, the use of BHA and BHT as food additives is controversial [44, 45].

During the 5-LOX assay viability of the leukocytes (Trypan blue exclusion) in the presence of isoflavonoids was 80–105% of the control. In the presence of BHT the viability decreased to 37% of

the control value. Some isoflavonoids were tested on P 388 leukemia in mice and were found, up to 240 mg/kg test animal, not to exert any cytotoxic effect [46]. The bioavailability of a physiologically active compound tested *in vitro* is one of the factors determinative for a possible therapeutic use of such compounds. Few data are available on the metabolism and pharmacokinetics of isoflavonoids [9, 47] (for review see Ref. 48). These studies concerned distribution, storage and elimination of isoflavonoids. We have investigated the absorption of some isoflavonoids on isolated intestinal segments of the rat (unpublished data).

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Einsatz von Flavonoiden und Isoflavonoiden zur Kontrolle von Lagerpilzen der Gattung *Aspergillus* an Leguminosen

Von
M. Weidenbörner*, H. Hindorf und H. C. Jha

(Eingegangen am 10. 1. 1990)

Summary

Control of Storage Fungi of the Genus *Aspergillus* on Legumes with Flavonoids and Isoflavonoids

In the following investigation natural products like flavonoids and isoflavonoids and their derivatives were screened for their fungicidal effects against storage fungi of the genus *Aspergillus* on seeds of soybean (*Glycine max* L.) and bean (*Phaseolus vulgaris* L.). A high reduction in fungal infestation in bean seeds was achieved with the mixtures flavanone/6,7-Dihydroxy-4'-methoxyisoflavan 2 % a. i. and 6,7-Dihydroxy-4'-methoxyisoflavan/6,7-Dihydroxy-3'-methylisoflavan 2 % a. i. after three weeks with 87.9 % and 87.1 %, respectively. A significant reduction of infestation in soybeans was achieved with the combination of the isoflavans 6,7-Dihydroxy-4'-methoxyisoflavan/6,7-Dihydroxy-3'-methylisoflavan 2 % a. i. after two weeks with 69.9 %. Benomyl 1 % a. i. caused similar inhibition rates in both seed treatments.

Einleitung

In den Ländern der Tropen und Subtropen kommt dem Vorratsschutz aufgrund der dortigen klimatischen Gegebenheiten eine entscheidende Bedeutung zu. Während sich die weltweiten Lagerverluste nach Schätzungen der FAO (1987) auf jährlich 5 % belaufen, können nach Neergaard (1977) die Verluste im Lager in den Entwicklungsländern bis zu 30 % betragen. Neben Schädlingen, Bakterien und Viren bereiten vor allem Lagerpilze der Gattungen *Aspergillus* und *Penicillium* große Probleme (Jain et al., 1983; Umechuruba, 1985; El-Kady et al., 1986). Gruppen oder Arten dieser Gattungen wachsen ohne frei verfügbares Wasser schon bei einer äußerst geringen Samenfeuchtigkeit (Christensen, 1972). Ihr Wachstum führt zum Verderb der Samen und schafft die Voraussetzungen für das Wachstum anderer Mikroorganismen (Christensen und Kaufmann, 1965). Durch die Schwächung und das Absterben des Embryos kann die Keimfähigkeit

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higkeit befallener Samen stark vermindert sein (Ellis et al., 1974), so daß eine ausreichende Menge an Saatgut für die nächste Aussaat nicht zur Verfügung steht. Gelangen verdorbene Samen noch zum Verzehr, können die in den Samen enthaltenen Mykotoxine zu schwerwiegenden organischen Schäden bei Mensch und Tier führen (Wogan, 1986; Shank, 1976; Lyr, 1985; Krämer, 1987).

Besondere Aufmerksamkeit erfordert der Lagerpilzbefall bei eiweißreichen Samen. Durch ein ausreichendes Angebot von Leguminosensamen, die 20–40 % Eiweiß und zudem noch beträchtliche Mengen an Kohlenhydraten enthalten, könnte die angespannte Ernährungslage in den Entwicklungsländern teilweise entschärft werden (Frankie, 1981). Ansätze und Möglichkeiten zur Bekämpfung von Lagerpilzen sind vorhanden (Brown et al., 1975; Ellis et al., 1975; Madaan und Chohan, 1978; Malini et al., 1983a; Vanangamudi und Karivaratharaju, 1986; Ceynowa und Schulz, 1989), doch haben chemische, physikalische als auch biologische Verfahren Nachteile hinsichtlich ihrer Effektivität und/oder Toxizität (Malini et al., 1983b; Agnihotri, 1983). Auch neue Erkenntnisse über Rückstandsprobleme, Toxizität von Pflanzenschutzmitteln und als Folge davon verschärfte Verordnungen im Bereich des Pflanzenschutzmitteleinsatzes (Anonym, 1988), machen die Entwicklung anderer Verfahren unumgänglich.

Eine Alternative könnte der Einsatz pflanzlicher Naturstoffe wie Phytoalexine sein, die erstmals von Müller und Börger (1940) als Abwehrstoffe der Kartoffel gegen *Phytophthora infestans* diskutiert wurden. Diese pflanzlichen Sekundärmetaboliten sind hoch aktiv und wirken sehr spezifisch (Cruickshank und Perrin, 1971; Fraile et al., 1982; Weidenböcker et al., 1987). Rückstandsprobleme dürften aufgrund ihres Naturstoffcharakters nicht auftreten. Dies wird durch Untersuchungen über die Transformation von Naturstoffen u. a. durch pflanzliche Zellsuspensionen belegt (Barz und Grisebach, 1966; Berlin und Barz, 1971; Berlin, 1972; Berlin et al., 1974; Kallage, 1984). Die hier aufgezeigte Problematik war der Anlaß, mit Naturstoffen und deren Derivaten eine alternative Samenbehandlung durchzuführen.

Material und Methoden

Die fungizide Wirksamkeit von Flavonoiden und Isoflavonoiden in Flüssigkeitsversuchen gegen *Aspergillus* spp. ist bekannt (Weidenböner et al., 1989a; Weidenböner et al., 1989b). Die eingesetzten Flavonoide A und B waren beide unsubstituiert und unterschieden sich nur im Hydrierungsgrad (Abb. 1). Die drei getesteten Isoflavonoide C, D und E besaßen zwei Hydroxylgruppen (Position 6 und 7), eine Methoxygruppe (Position 4') oder eine Methylgruppe (Position 3') (Abb. 1). Die in den Untersuchungen verwendeten Flavonoide und Isoflavonoide lagen hochgereinigt in Pulverform vor. Mit Ausnahme der Flavonoide waren die Substanzen im Institut für Physiologische Chemie der Universität Bonn synthetisch hergestellt worden. Die Authentizität der Substanzen wurde durch physikalisch-chemische Methoden eindeutig festgestellt (JHA, pers. Mitt.). Die Flavonoide wurden bei der Firma Merck, Darmstadt, erworben. Die Wirksamkeit dieser Substanzen wurde mit der des konventionellen Fungizids Benomyl F verglichen. Für die Samenbehandlung standen Samen der Sojabohne (*Glycine max* L.) und der Buschbohne (*Phaseolus vulgaris* L.) zur Verfügung.

Unter sterilen Bedingungen wurden in ein 50 ml Becherglas, das auf einem Magnetrührer stand, ein Rührmagnet sowie 30 Samen (bei 4 Wiederholungen) einer Samenart

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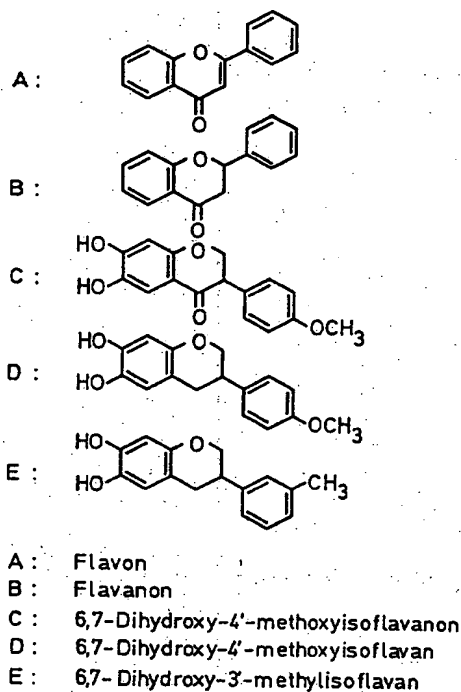


Abb. 1: Struktur der getesteten Flavonoide und Isoflavonoide

gegeben. Die für die Behandlung notwendige Substanzmenge, 1 bzw. 2 GW %/V aktive Substanz (a. S.) (Dhingra und Muchovej, 1982), wurde im Fall der Sojabohne in 7 ml Aceton bzw. Ethanol und bei der Buschbohne in 10 ml Aceton gelöst. Hierdurch konnte eine vollständige Bedeckung der Samen mit dem jeweiligen Lösungsmittel während der Behandlung erreicht werden. Nach Zugabe der gelösten Substanz in das Becherglas wurde der Magnetrührer auf mittlerer Umdrehungszahl eingeschaltet. Die Samen verblieben für 30 Minuten unter ständigem Rühren in dem Lösungsmittel, das anschließend abgossen wurde. Zur Verdampfung des noch an den Samen anhaftenden Lösungsmittels wurden diese für 30 Minuten in eine offene Glaspetrischale gelegt. Anschließend erfolgte das Auslegen von jeweils 3 Samen/Petrischale auf Salz-Malzextrakt Agar (SMA 10 % NaCl)-Platten. Der NaCl-Zusatz verhindert nach Mislivec und Bruce (1977) das Bakterienwachstum, hemmt die schnelle Entwicklung von *Mucorales*-Arten sowie anderer Pilze und unterbindet die Keimung der Samen. Auf diese Weise konnten zum Versuchsende alle aufgetretenen Pilze bis zur Gattung bzw. Gruppe bestimmt werden. Die Bestimmung der Mykoflora nach der Behandlung sollte zeigen, ob die Substanzen einzeln oder im Gemisch einheitlich das Wachstum aller oder nur bestimmter Pilze am oder im Samen verhindern.

Die Bonitur in befallene und nicht befallene Samen erfolgte im Abstand von 7 Tagen bis zum 21. Tag nach dem Auslegen im Fall der Buschbohnen. Die Versuche der Sojabohnen konnten nur bis zum 14. Tag ausgewertet werden, da schnell wachsende Pilze der *A. niger*- bzw. *A. glaucus*-Gruppe auftraten. Eine sichere Bonitur war deshalb nur bis zu dem o. a. Boniturtermin möglich. Zudem konnte bei den Sojabohnen nach 2 Wochen, bei den Buschbohnen nach 3 Wochen, ein Befallsanstieg nicht mehr festgestellt werden.

Die ermittelten Befallsdaten wurden varianzanalytisch ausgewertet. Die verschiedenen Behandlungen einer Samenart stellten eine Versuchseinheit dar und wurden einzeln verrechnet. Durch die Berechnung der Grenzdifferenzen konnten die Einzeldifferenzen, die in einer Versuchseinheit auftraten, statistisch abgesichert werden.

Zur Ermittlung der Keimraten nach einer Naturstoffbehandlung wurden jeweils 30 Samen der Sojabohne und der Buschbohne nach der oben beschriebenen Methode ausgelegt. Die Substanzen Flavon (A), Flavanon (B), 6,7-Dihydroxy-4'-methoxyisoflavan (D) und 6,7-Dihydroxy-3'-methylisoflavan (E) wurden in der Konzentration 1 % a. S. in Ethanol gelöst. Nach dem Auslegen der behandelten Samen in eine feuchte Kammer erfolgte die Bonitur der Keimraten nach 7 Tagen.

Zur Bestimmung des Feuchtegehaltes der Sojabohnen und Buschbohnen wurden die Samen auf SMA ausgelegt. Nach einer Woche wurden 4–5 g einer Samenart entnommen, in eine zuvor gewogene Glaspetrishale gegeben und anschließend gewogen. Nach erfolgter Trocknung der Samen bei 130 °C für eine Stunde in einem Muffelofen und Abkühlen in einem Exsikkator wurde die Petrishale mit Inhalt nach Roberts und Roberts (1972) erneut gewogen. Der Feuchtegehalt der Samen konnte anhand folgender Formel ermittelt werden:

$$(M_2 - M_3) \times \frac{100}{(M_2 - M_1)}$$

M_1 : Gewicht der leeren Petrishale

M_2 : Gewicht der Petrishale mit Inhalt

M_3 : Gewicht der Petrishale mit Inhalt nach dem Trocknen

Ergebnisse

Bei der Behandlung der Buschbohnen besaß Flavon (A) mit 71.1 % bzw. 80.0 % eine sehr hohe befallsreduzierende Wirkung in der Konzentration 1 % bzw. 2 % a. S. nach einer Woche. In der Konzentration 1 % a. S. verringerte Flavanon (B) den Befall der Buschbohnen um 48.9 %, in der Konzentration 2 % a. S. um 72.2 %. Das 6,7-Dihydroxy-4'-methoxyisoflavanon (C) verursachte in der Konzentration 1 % a. S. eine Befallsreduzierung um 45.6 %. Das entsprechende Isoflavan (D) reduzierte in der Konzentration 1 % a. S. den Befall um 56.7 % und in der Konzentration 2 % a. S. um 74.4 %. Mit genau 60.0 % hemmte das 6,7-Dihydroxy-3'-methylisoflavan (E) den Pilzbefall der Buschbohnen in der Konzentration 1 % a. S. Hochwirksam waren 2 %ige Mischungen von A/D, B/D sowie D/E mit über 90 % Befallsreduktion im Fall der Buschbohne nach einer Woche. Auch Benomyl (F) reduzierte den Pilzbefall zu über 90 % allerdings in der Konzentration 1 %. Das Vergleichsfungizid Benomyl (F) 1 % a. S. war die wirksamste Substanz bei der Behandlung der Sojabohnen (Aceton) mit 81.4 % nach einer Woche.

Die Wirkung vom Isoflavanon D/E 2 % a. S. mit 44.9 (Ethanol) waren nach der E frei. Die Mischung A/E 2 Benomyl (F) 1 % a. S. be 23.2 % auf. Wirksam war 54.5 % in der Konzentration

Tab. 1: Prozent befallsfrei

Substanzen

A	1%
A	2%
B	1%
B	2%
C	1%
D	1%
D	2%
E	1%
E	2%
A/B	2%
A/C	2%
A/D	2%
A/E	2%
B/D	2%
D/E	2%
F	1%

* GD 5 %; A = Aceton; E = Et

In der zweiten Woche Flavanon (B) und 6,7-Dihydroxy-4'-methoxyisoflavanon (C) stark nach. Dagegen wie Benomyl (F) auch in Aceton zeigte sich eine Benomyl (F) 1 % a. S. betrug, nahm der Befall der zu. Auch bei den Sojabohnen (F) mit 16.6 % am Der Wirkungsabfall der ü

Die Wirkung vom Isoflavan D 2 % a. S. betrug 59.3 % gefolgt von der Isoflavankombination D/E 2 % a. S. mit 44.9 % und 38.5 % Isoflavan D 1 % a. S. 78.6 % der Sojabohnen (Ethanol) waren nach der Behandlung mit den beiden Isoflavanen D/E 2 % a. S. befallsfrei. Die Mischung A/E 2 % a. S. verursachte nur eine Befallsreduzierung um 40.2 %. Benomyl (F) 1 % a. S. behandelte Samen wiesen dagegen nur einen Befallsgrad von 23.2 % auf. Wirksam waren das Isoflavan D mit 67.9 % und das Isoflavan (E) mit 54.5 % in der Konzentration 2 % a. S. (Tab. 1).

Tab. 1: Prozent befallsfreier Samen nach einer 30 minütigen Wirkstoffbehandlung in Aceton bzw. Ethanol nach einer Woche

Substanzen	Buschbohnen 30'A	Samenarten Sojabohnen 30'A	Sojabohnen 30' E
A 1%	-71.1*	-1.7	—
A 2%	-80.0*	—	-16.1*
B 1%	-48.9*	-1.7	—
B 2%	-72.2*	—	-7.1
C 1%	-45.6*	-5.9	—
D 1%	-56.7*	-38.5*	—
D 2%	-74.4*	-59.3*	-67.9*
E 1%	-60.0*	—	-36.6*
E 2%	—	—	-54.5*
A/B 2%	—	-9.3	-12.5
A/C 2%	—	-11.0	—
A/D 2%	-90.0*	—	—
A/E 2%	—	—	-40.2*
B/D 2%	-95.6*	—	—
D/E 2%	-93.3*	-44.9*	-78.6*
F 1%	-93.3*	-81.4*	-76.8*

* GD 5 %; A = Aceton; E = Ethanol

In der zweiten Woche ließ die Wirksamkeit einzelner Substanzen wie Flavon (A), Flavanon (B) und 6,7-Dihydroxy-4'-methoxyisoflavanon (C) in der Buschbohnenbehandlung stark nach. Dagegen zeigte sich, daß die o. a. Mischungen A/D, B/D und D/E sowie Benomyl (F) auch noch in der zweiten Woche wirksam waren. Bei den Sojabohnen (Aceton) zeigte sich eine ähnliche Tendenz (Tab. 2). Während aber der Befallsanstieg Benomyl (F) 1 % a. S. behandelte Samen von der ersten zur zweiten Woche 16 % betrug, nahm der Befall der Samen der Isoflavankombination D/E 2 % a. S. nur um 6.8 % zu. Auch bei den Sojabohnen (Ethanol) war der Befallsanstieg in der Benomyl-Behandlung (F) mit 16.6 % am höchsten. Die Wirksamkeit Isoflavans D ließ um 11.3 % nach. Der Wirkungsabfall der übrigen Substanzen war gering (Tab. 2).

Tab. 2: Prozent befallsfreier Samen nach einer 30 minütigen Wirkstoffbehandlung in Aceton bzw. Ethanol nach zwei Wochen

Substanzen	Buschbohnen 30'A	Samenarten Sojabohnen 30'A	Sojabohnen 30'E
A 1%	-24.6*	-0.8	—
A 2%	-43.9*	—	-11.5
B 1%	-5.3	0	—
B 2%	-14.6	—	-5.3
C 1%	-30.7*	-3.4	—
D 1%	-43.0*	-31.4	—
D 2%	-66.7*	-49.2*	-56.6*
E 1%	-53.5*	—	-31.0*
E 2%	—	—	-51.3*
A/B 2%	—	-5.1	-10.6
A/C 2%	—	-5.9	—
A/D 2%	-71.9*	—	—
A/E 2%	—	—	-35.4*
B/D 2%	-88.6*	—	—
D/E 2%	-87.7*	-38.1*	-69.9*
F 1%	-93.0*	-64.4	-60.2*

* = GD 5%; Fettdruck = der aufgeführte Wert wurde aufgrund der abweichenden Streuung der Einzelwerte in dieser Behandlung nicht varianzanalytisch verrechnet; A = Aceton; E = Ethanol

Tab. 3: Prozent befallsfreier Samen nach einer 30 minütigen Wirkstoffbehandlung in Aceton nach drei Wochen

Substanzen	Samenart Buschbohnen 30'A
A 1%	-22.4*
A 2%	-42.2*
B 1%	-6.0
B 2%	-11.5
C 1%	-30.2*
D 1%	-43.1*
D 2%	-65.5*
E 1%	-52.6*
A/D 2%	-70.7*
B/D 2%	-87.9*
D/E 2%	-87.1*
F 1%	-92.2*

* = GD 5%; A = Aceton

Der Befallsgrad der Buschbohnen in der dritten Woche nur unwesentlich.

Den Einfluß der verschiedenen Samenarten zeigen die Tabellen 4 und 5. Die Kontrollvariante ein hoher Befallsgrad von *Alternaria* spp. festgestellt. Dies sowie Benomyl (F) 1% a. S. r. wurde festgestellt (Tab. 4).

Tab. 4: Pilzspektrum der Buschbohnen nach einer 30 minütigen Wirkstoffbehandlung (A = Aceton)

Pilze	K
<i>A. niger</i>	1
<i>A. flavus</i>	0
<i>A. glaucus</i>	3
<i>A. nidulans</i>	3
<i>A. ochraceus</i>	1
<i>A. terreus</i>	3
<i>A. fumigatus</i>	0
<i>A. ustus</i>	5
<i>A. wentii</i>	1
<i>Penicillium</i> spp.	1
<i>Cladosporium</i> spp.	103
<i>Alternaria</i> spp.	23
<i>Acremonium</i> spp.	0
<i>Fusarium</i> spp.	1
<i>Phoma</i> spp.	0
<i>Humicola</i> spp.	0
<i>Mortierella</i> spp.	0

<i>A. niger</i>
<i>A. glaucus</i>
<i>A. nidulans</i>
<i>A. ochraceus</i>
<i>A. terreus</i>
<i>A. ustus</i>
<i>Penicillium</i> spp.
<i>Cladosporium</i> spp.
<i>Alternaria</i> spp.
<i>Acremonium</i> spp.
<i>Fusarium</i> spp.
<i>Phoma</i> spp.
<i>Mortierella</i> spp.

andlung in

bohnen
10'E

11.5

-5.3

56.6*

31.0*

51.3*

10.6

35.4*

69.9*

60.2*

der Einzelwerte

andlung in

Der Befallsgrad der Buschbohnen veränderte sich verglichen mit der zweiten Woche in der dritten Woche nur unwesentlich (Tab. 3).

Den Einfluß der verschiedenen Behandlungen auf das Pilzspektrum der beiden Samenarten zeigen die Tabellen 4, 5 und 6. Im Fall der Buschbohnen wurde in der Kontrollvariante ein hoher Befallsgrad mit *Cladosporium* spp. und in geringerem Maße mit *Alternaria* spp. festgestellt. Die Substanzkombinationen A/D, B/D, D/E alle 2% a. S. sowie Benomyl (F) 1% a. S. reduzierten den Befall der Samen mit Pilzen dieser beiden Gattungen (Tab. 4).

Tab. 4: Pilzspektrum der Buschbohnen (120 Samen/Variante) nach einer Wirkstoffbehandlung (Aceton/30 Minuten) drei Wochen nach dem Auslegen

Pilze	K	Substanz					
		A 1 %	B 1 %	A 2 %	B 2 %	C 1 %	D 1 %
<i>A. niger</i>	1	1	1	0	1	0	1
<i>A. flavus</i>	0	0	3	1	0	0	0
<i>A. glaucus</i>	3	2	0	1	1	0	0
<i>A. nidulans</i>	3	0	1	0	0	3	0
<i>A. ochraceus</i>	1	2	0	0	0	0	0
<i>A. terreus</i>	3	4	3	6	5	0	4
<i>A. fumigatus</i>	0	0	0	0	1	0	0
<i>A. ustus</i>	5	3	3	1	2	2	0
<i>A. wentii</i>	1	0	0	0	0	0	0
<i>Penicillium</i> spp.	1	2	1	0	1	1	1
<i>Cladosporium</i> spp.	103	70	98	39	71	62	61
<i>Alternaria</i> spp.	23	15	21	17	12	28	23
<i>Acremonium</i> spp.	0	0	0	0	2	0	0
<i>Fusarium</i> spp.	1	2	0	2	0	0	2
<i>Phoma</i> spp.	0	0	0	0	0	1	0
<i>Humicola</i> spp.	0	0	0	1	0	0	0
<i>Mortierella</i> spp.	0	0	0	1	0	0	0

	D 2 %	E 1 %	A/D 2 %	B/D 2 %	D/E 2 %	F 1 %
<i>A. niger</i>	1	0	0	0	0	0
<i>A. glaucus</i>	0	1	1	1	0	0
<i>A. nidulans</i>	0	1	0	0	0	0
<i>A. ochraceus</i>	0	1	0	0	0	0
<i>A. terreus</i>	0	1	4	1	0	0
<i>A. ustus</i>	1	1	0	0	0	0
<i>Penicillium</i> spp.	0	0	0	0	1	0
<i>Cladosporium</i> spp.	26	40	1	4	6	0
<i>Alternaria</i> spp.	9	13	5	7	8	7
<i>Acremonium</i> spp.	1	1	0	0	0	1
<i>Fusarium</i> spp.	0	1	0	0	0	0
<i>Phoma</i> spp.	0	1	1	0	0	0
<i>Mortierella</i> spp.	0	0	0	0	0	1

Samen der Sojabohne (Aceton) waren in der Kontrolle stark mit *A. glaucus* und *Penicillium* spp. infiziert. Die Behandlung mit Benomyl (F) 1 % a. S. reduzierte das Auftreten dieser Pilze fast vollständig (Tab. 5). Das Isoflavan D 1 % a. S. minderte den Befall der Samen mit *Penicillium* spp. stark. In der Konzentration 2 % a. S. reduzierten das Isoflavan D und die Isoflavankombination D/F das Auftreten von *A. glaucus* um 50 %.

Tab. 5: Pilzspektrum der Sojabohnen (120 Samen/Variante) nach einer Wirkstoffbehandlung (Aceton/30 Minuten) zwei Wochen nach dem Auslegen

Pilze	K	Substanz			
		A 1 %	B 1 %	C 1 %	D 1 %
<i>A. niger</i>	0	0	0	0	1
<i>A. flavus</i>	0	1	0	2	1
<i>A. glaucus</i>	105	117	102	83	72
<i>A. ochraceus</i>	3	2	0	0	0
<i>A. restrictus</i>	2	4	0	2	1
<i>A. candidus</i>	14	10	24	13	5
<i>A. ustus</i>	0	0	0	1	0
<i>A. versicolor</i>	12	5	6	8	2
<i>A. flavipes</i>	0	2	0	0	0
<i>Penicillium</i> spp.	57	28	33	33	9
<i>Alternaria</i> spp.	6	10	6	8	5
<i>Acremonium</i> spp.	6	8	0	2	0
<i>Fusarium</i> spp.	2	3	0	0	0
<i>Mortierella</i> spp.	0	0	0	3	0
<i>Geotrichum</i> spp.	1	0	0	0	0
<i>Scopulariopsis</i> spp.	0	9	0	0	0
<i>Wallemia</i> spp.	2	2	1	0	2
	D 2 %	A/B 2 %	A/D 2 %	D/% 2 %	F 1 %
<i>A. niger</i>	1	0	0	0	0
<i>A. flavus</i>	0	0	1	0	0
<i>A. glaucus</i>	51	83	102	44	0
<i>A. restrictus</i>	1	6	0	1	0
<i>A. candidus</i>	1	8	7	2	0
<i>A. versicolor</i>	1	8	10	4	0
<i>A. flavipes</i>	0	5	0	0	0
<i>Penicillium</i> spp.	24	32	34	28	1
<i>Alternaria</i> spp.	10	6	10	6	19
<i>Acremonium</i> spp.	0	1	0	0	0
<i>Fusarium</i> spp.	0	0	2	0	0
<i>Mortierella</i> spp.	0	0	1	1	2
<i>Scopulariopsis</i> spp.	0	1	0	0	3
<i>Phialophora</i> spp.	0	0	0	0	2
<i>Wallemia</i> spp.	0	0	2	0	39

Tab. 6: Pilzspektrum (Kontrolle) und Wirkungstoffbehandlung (Aceton)

Pilze	K
<i>A. flavus</i>	2
<i>A. glaucus</i>	91
<i>A. restrictus</i>	3
<i>A. candidus</i>	4
<i>A. nidulans</i>	0
<i>A. ochraceus</i>	0
<i>A. versicolor</i>	14
<i>Penicillium</i> spp.	32
<i>Alternaria</i> spp.	14
<i>Acremonium</i> spp.	3
<i>Fusarium</i> spp.	1
<i>Mortierella</i> spp.	1
<i>Scopulariopsis</i> spp.	1
<i>Wallemia</i> spp.	4

<i>A. flavus</i>
<i>A. glaucus</i>
<i>A. restrictus</i>
<i>A. candidus</i>
<i>A. nidulans</i>
<i>A. versicolor</i>
<i>A. flavipes</i>
<i>Penicillium</i> spp.
<i>Alternaria</i> spp.
<i>Acremonium</i> spp.
<i>Mortierella</i> spp.
<i>Scopulariopsis</i> spp.
<i>Wallemia</i> spp.

Auch mit Ethanol behandelte und *Penicillium* spp. infizierte Samen und die Isoflavankombination D/F waren wirksam, um den Befall der Samen zu senken (Tab. 6).

Eine Naturstoffbehandlung (Tab. 6).

Nach dem Auslegen der Buschbohnen 29 % und bei

Tab. 6: Pilzspektrum der Sojabohne (120 Samen/Variante) nach einer Wirkstoffbehandlung (Aceton/30 Minuten) zwei Wochen nach dem Auslegen

Pilze	K	Substanz				
		A 2 %	B 2 %	D 2 %	E 1 %	E 2 %
<i>A. flavus</i>	2	0	0	0	2	1
<i>A. glaucus</i>	91	81	81	31	56	38
<i>A. restrictus</i>	3	0	1	1	3	2
<i>A. candidus</i>	4	4	8	1	3	3
<i>A. nidulans</i>	0	0	1	0	0	0
<i>A. ochraceus</i>	0	1	0	0	0	0
<i>A. versicolor</i>	14	4	14	0	6	3
<i>Penicillium</i> spp.	32	43	43	12	25	11
<i>Alternaria</i> spp.	14	7	12	5	7	14
<i>Acremonium</i> spp.	3	2	1	0	0	0
<i>Fusarium</i> spp.	1	0	1	0	1	0
<i>Mortierella</i> spp.	1	0	1	1	0	0
<i>Scopulariopsis</i> spp.	1	0	1	0	0	0
<i>Wallemia</i> spp.	4	4	4	0	4	3
		A/B 2 %	A/E 2 %	D/E 2 %	F 1 %	
<i>A. flavus</i>		1	1	0	0	
<i>A. glaucus</i>		73	51	21	1	
<i>A. restrictus</i>		3	1	1	0	
<i>A. candidus</i>		2	2	1	0	
<i>A. nidulans</i>		1	0	0	0	
<i>A. versicolor</i>		8	1	1	0	
<i>A. flavipes</i>		0	1	0	0	
<i>Penicillium</i> spp.		43	21	9	0	
<i>Alternaria</i> spp.		5	4	5	40	
<i>Acremonium</i> spp.		0	3	1	0	
<i>Mortierella</i> spp.		1	0	1	2	
<i>Scopulariopsis</i> spp.		0	2	0	0	
<i>Wallemia</i> spp.		3	1	0	17	

Auch mit Ethanol behandelte Sojasamen waren in der Kontrolle stark mit *A. glaucus* und *Penicillium* spp. infiziert. Es zeigte sich, daß die Isoflavane D und E 2 % a. S. und die Isoflavankombination D/E 2 % a. S. den Befall mit diesen beiden Lagerpilzen effektiv senken konnten. Während Benomyl (F) 1 % a. S. gegen diese Pilze hoch wirksam war, wurde ein starker Befallsanstieg mit *Alternaria* spp. und *Wallemia* spp. festgestellt (Tab. 6).

Eine Naturstoffbehandlung der Samen führte nicht zu verringerten Keimraten (Tab. 7).

Nach dem Auslegen der Samen auf SMA betrug die Samenfeuchte am 7. Tag bei den Buschbohnen 29 % und bei den Sojabohnen 38 %.

laucus und Pe-
rte das Auftre-
rte den Befall
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39

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Tab. 7: Anzahl gekeimter Samen nach einer Naturstoffbehandlung nach 7 Tagen

Substanz	Buschbohne	Sojabohne
K	30	29
A 1 %	29	29
B 1 %	30	30
D 1 %	—	30
E 1 %	30	28

Diskussion

Lagerpilze der Gattung *Aspergillus* und *Penicillium* führen zum Verderb und zur Mykotoxinkontamination von eingelagerten Samen. In einigen Ländern betragen die jährlichen Verluste bis zu 30 % der Ernte (Neergaard, 1977). Der häufige Befall der Samen mit Primärbesiedlern aus der *A. glaucus*-Gruppe (Raper und Fennell, 1965; Kennedy, 1964; Mislivec und Bruce, 1977; Onesirosan, 1982; Weidenbörner und Hindorf, 1989) und Toxinbildnern wie *A. flavus* und *A. petrakii* (Hesseltine et al., 1966; Hesseltine et al., 1972) stellt die dortige Landwirtschaft vor große Probleme. Physikalische, chemische und biologische Verfahren sind zur Kontrolle von Lagerpilzen entwickelt worden (Feuell, 1966; Ashworth et al., 1968; Schroeder et al., 1968; Brown et al., 1975; Pathak et al., 1981; Bilgrami et al., 1982; Malini et al., 1983b; Reddy et al., 1983). Ihre Anwendung und/oder Effektivität ist aber aus verschiedenen Gründen problematisch. Eine Alternative könnte der Einsatz von Naturstoffen aus der Klasse der Flavonoide/Isoflavonoide sein, deren antimikrobielle Eigenschaften bekannt sind (Cruickshank und Perrin, 1971; Kaplan und Thomason, 1980; Bull, 1981; Pett und Osman, 1982).

Eine Infektion der behandelten Samen wurde zuerst in der Hilumregion sichtbar, die eine natürliche Bruchstelle zwischen dem Samen und dem Funiculus darstellt (Gunn, 1981; Strasburger et al., 1983). Das Hilum kann neben der Mikropyle als Hauptinfektionsstelle für Lagerpilze angesehen werden, soweit keine Risse oder Brüche in der Samenschale vorhanden sind (Lopez und Christensen, 1962). Ein Pilzwachstum auf der Samenoberfläche wurde dagegen seltener festgestellt. Die teilweise hohen Befallsreduktionen der Samen durch eine Naturstoffbehandlung zeigen, daß die Substanzen nicht nur eine Oberflächen desinfizierende Wirkung besitzen. Es ist davon auszugehen, daß die Stoffe in Verbindung mit bestimmten organischen Lösungsmitteln bis in die Testa gelangen. Eine „Desinfektion“ der Samenoberfläche war erwartet worden, da die fungizide Wirkung dieser Substanzen gegen Myzel und Sporen bekannt ist (Cruickshank und Perrin, 1961; Tomiyama et al., 1968; van Etten und Bateman, 1971; McCance und Drysdale, 1975; Smith, 1976; Krämer et al., 1984; Weidenbörner et al., 1989c). Obwohl aufgrund der Untersuchungen von Meyer und Mayer (1971), Tao et al. (1974), Royse et al. (1975), Ellis et al. (1976), Muchovej und Dhingra (1979b) sowie Muchovej und Dhingra (1980) von einem Transport der Substanzen bis in die Samenschale ausgegangen werden konnte, ist dieser mögliche Effekt doch etwas überraschend. Nach Dhingra und Muchovej (1980) werden nämlich Thiophanatmethyl und

RH 2161 in Verbindung transportiert.

In den Buschbohnen (A) 1 % a. S. und von F Konzentrationserhöhung z Abfallen der Wirksamkeit dert werden (Tab. 3). Ver flavonons (C) 1 % a. S. w Ring C und die aplanare 1969; van Etten, 1970. Rolle zu spielen. Es wär stanz in die Samenschale fungizide Wirkung in der die Wirkung der Substanzen nach 7 Tagen waren 52,6 % der Buschbohnenbefallungsmittel (Nandi und lundung auf beispielsweise 4 weitere Untersuchungen i besten Ergebnisse erzielt, 2; 3). Auffällig ist hier, d van (B/D) 2 % a. S. imm flavans mit Flavon (A/D von (A) stets wirksamer z die Ursache sein. Nur di 3'-methylisoflavan (D/E fallsreduktionen. Die s Wirksamkeit dieser beid al.; 1987. und 1989a) be Sojabohne (Aceton) war handelten Samen befalls im Vergleich zur Kombi methylisoflavan (D/D) z toxischen in vitro Wirk und 1989a), war eine i worden. Gerade diese Möglicherweise wird ne Aceton in die Samensch Der Transport einer sungsmittel ab (Tao et gra und Muchovej, Ethanol bei der Sojabot jabohnen (Ethanol) fik Befallsreduktionen. Die 3'-methylisoflavan (D/f 1; 2). Es scheint, daß i

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In den Buschbohnenversuchen konnte eine nachlassende Wirksamkeit von Flavon (A) 1 % a. S. und von Flavanon (B) 1 % a. S. beobachtet werden (Tab. 1, 2, 3). Eine Konzentrationserhöhung auf 2 % a. S. führte jedoch zu einer verbesserten Wirkung. Ein Abfallen der Wirksamkeit bis zum 21sten Tag konnte aber auch hierdurch nicht verhindert werden (Tab. 3). Verglichen mit der Wirksamkeit des 6,7-Dihydroxy-4'-methoxyisoflavanons (C) 1 % a. S. war das Isoflavan (D) 1 % a. S. aktiver. Der vollständig hydrierte Ring C und die aplanare Molekülstruktur von Isoflavanen (Perrin und Cruickshank, 1969; van Etten, 1976; van Etten und Pueppke, 1976) scheinen eine gewissen Rolle zu spielen. Es wäre möglich, daß diese Molekülstruktur das Eindringen der Substanz in die Samenschale erleichtert. Eine erhöhte Konzentration und damit eine erhöhte fungizide Wirkung in der Testa wären die Folgen. In der Konzentration 2 % a. S. konnte die Wirkung der Substanz um weitere 20 % gesteigert werden (Tab. 1, 2, 3). Nach 21 Tagen waren 52.6 % der mit 6,7-Dihydroxy-3'-methylisoflavan (E) 1 % a. S. behandelten Buschbohnenansamen befallsfrei. Inwiefern andere oder Mischungen verschiedener Lösungsmittel (Nandi und Fries, 1976) oder eine Temperaturerhöhung bei der Behandlung auf beispielsweise 40 °C die Effektivität der Substanzen steigern können, müssen weitere Untersuchungen klären. Bei der Behandlung der Buschbohnenansamen wurden die besten Ergebnisse erzielt, wenn zwei Substanzen kombiniert zum Einsatz kamen (Tab. 1, 2, 3). Auffällig ist hier, daß die Kombination Flavanon/6,7-Dihydroxy-4'-methoxyisoflavan (B/D) 2 % a. S. immer eine höhere Aktivität als die Substanzkombination dieses Isoflavans mit Flavon (A/D) 2 % besaß (Tab. 1, 2, 3). Einzeln angewendet war nämlich Flavon (A) stets wirksamer als Flavon (B). Synergistische Effekte zwischen B und D könnten die Ursache sein. Nur die Mischung 6,7-Dihydroxy-4'-methoxyisoflavan/6,7-Dihydroxy-3'-methylisoflavan (D/E) 2 % a. S. und Benomyl (F) 1 % a. S. erzielten noch höhere Befallsreduktionen. Die schon in früheren Untersuchungen festgestellte hohe fungizide Wirksamkeit dieser beiden Isoflavane unter in vitro Bedingungen (Weidenbörner et al., 1987 und 1989a) bestätigten sich auch in diesen Versuchen. Bei den Versuchen der Sojabohne (Aceton) waren nach 14 Tagen noch 64.4 % der Benomyl (F) 1 % a. S. behandelten Samen befallsfrei. Das 6,7-Dihydroxy-4'-methoxyisoflavan (D) 2 % a. S. zeigte im Vergleich zur Kombination 6,7-Dihydroxy-4'-methoxyisoflavan und 6,7-Dihydroxy-3'-methylisoflavan (D/D) 2 % a. S. eine höhere Wirkung (Tab. 1, 2). Aufgrund der fungitoxischen in vitro Wirkung der beiden Isoflavane D und E (Weidenbörner et al., 1987 und 1989a), war eine stärkere Wirksamkeit der Kombination D/E 2 % a. S. erwartet worden. Gerade diese Wirkstoffkombination war im Buschbohnenversuch hoch aktiv. Möglicherweise wird nur eine geringe Menge dieser Isoflavanmischung in Verbindung mit Aceton in die Samenschale der Sojabohne transportiert.

Der Transport einer Chemikalie in die Samenschale hängt u. a. vom verwendeten Lösungsmittel ab (Tao et al., 1974; Muchovej und Dhingra, 1979a und 1980; Dhingra und Muchovej, 1982). Aus diesem Grund wurde außer Aceton auch 96 %iges Ethanol bei der Sojabohnenbehandlung eingesetzt. Die Isoflavonoid-Behandlung der Sojabohnen (Ethanol) führte im Vergleich mit dem Versuch Sojabohne (Aceton) zu höheren Befallsreduktionen. Die Kombination 6,7-Dihydroxy-4'-methoxyisoflavan/6,7-Dihydroxy-3'-methylisoflavan (D/E) 2 % a. S. war sogar wirksamer als Benomyl (F) 1 % a. S. (Tab. 1, 2). Es scheint, daß Isoflavonoide in Verbindung mit Ethanol verstärkt in die Samen-

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Verwendung dieser Substanzen als Ersatz für konventionelle Fungizide möglich machen und somit sowohl das Nahrungsangebot als auch die für die nächste Aussaat benötigte Saatgutmenge in den betroffenen Entwicklungsländern garantieren.

Zusammenfassung

In der vorliegenden Untersuchung wurde die fungizide Wirkung von Naturstoffen, Flavonoiden und Isoflavonoiden sowie deren Derivate, auf Lagerpilze der Gattung *Aspergillus* anhand einer Samenbehandlung der Sojabohne (*Glycine max* L.) und der Buschbohne (*Phaseolus vulgaris* L.) getestet. Es zeigte sich, daß die Kombinationen Flavanon/6,7-Dihydroxy-4'-methoxyisoflavan 2 % a. S. und 6,7-Dihydroxy-4'-methoxyisoflavan/6,7-Dihydroxy-3'-methylisoflavan 2 % a. S. nach drei Wochen den Pilzbefall der Buschbohnen um 87,9 % bzw. 87,1 % reduzierten. Im Fall der Sojabohne konnte eine Hemmung des Pilzbefalls um 69,9 % durch die Kombination 6,7-Dihydroxy-4'-methoxyisoflavan/6,7-Dihydroxy-3'-methylisoflavan 2 % a. S. nach zwei Wochen erzielt werden. Benomyl 1 % a. S. behandelte Samen wiesen ähnlich niedrige Befallswerte auf.

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Zwei Jahre nach Er-
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Bandes erschienen die
zweite Band über Feldfrüchte
welcher bereits im
zweiten Band erschienen.
Die beiden Bände sind
weltwirtschaftlich inter-
essant und die Gemüsearten
neue Gemüsearten, Kartoffel
Verbreitung vermutet
werden, Gemüsearten und
Verbreitung finden insge-
samt sich Arten der Gattung
Cucumis, *Cichorium*, *Cichorium*,
Schizachyrium, *Schizachyrium*,
Echinochloa, *Lotus*, *Corn*.

Einleitend zu jedem
heutigen Verbreitungs-
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ANTIFUNGAL ACTIVITY OF ISOFLAVONOIDS IN DIFFERENT REDUCED STAGES ON *RHIZOCTONIA SOLANI* AND *SCLEROTIUM ROLFSSII*

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Key Word Index—*Rhizoctonia solani*; *Sclerotium rolfssii*; isoflavonoids; antifungal activity; structure-activity relationships.

Abstract—Two naturally occurring isoflavones, genistein and biochanin A, and their dihydroderivates (isoflavanones) as well as nine perhydrogenated isoflavones (isoflavans) were tested for their effects on mycelial growth of the two soil borne fungi *Rhizoctonia solani* and *Sclerotium rolfssii*. All the isoflavonoids of the biochanin A series showed high antifungal activity. Genistein isoflavan and the other isoflavans with two hydroxyl groups and one methoxy group were fungitoxic, while isoflavans with two or three methoxy groups were almost inactive.

INTRODUCTION

The effects of isoflavonoids on microorganisms have been investigated by several groups of workers [1-5]. It has been established that some of them possess strong antimicrobial activities [6-9]. However, less information is available regarding the structure-activity relationship of these compounds. In continuation of our previous work [1, 6, 10, 11] further data are presented in this publication.

RESULTS AND DISCUSSION

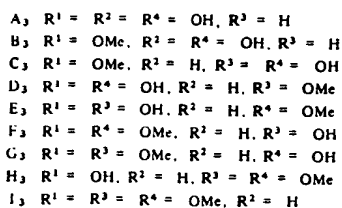
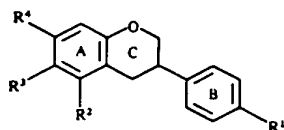
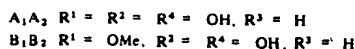
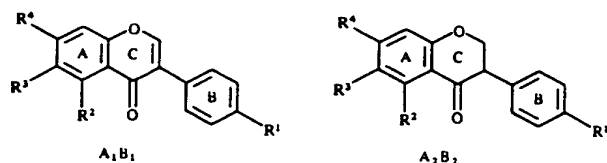
In the A_{1-3} series the isoflavan A_3 was the most effective substance inhibiting mycelial growth of *R. solani* to 79.5% and *S. rolfssii* to 91.6% at a concentration of 0.8 mM. In contrast to the A_{1-3} series, in the B_{1-3} series all the isoflavonoids showed high antifungal activity. At the highest concentration tested (0.8 mM) these isoflavonoids inhibited the growth of *R. solani* and *S. rolfssii* in the range of 70-80% and about 90% respectively. The antifungal activity of the isoflavan C_3 at concentrations of 0.2 and 0.8 mM was also high. *R. solani* was inhibited up to 80% and *S. rolfssii* to 91% at both these concentrations. The isoflavans D_3 and E_3 caused significant inhibitions of mycelial growth only in the highest concentration (0.8 mM). The inhibitory effects of both towards *S. rolfssii* amounted to 94%. The other four isoflavans, F_3 - I_3 , possessed low antifungal activity. A maximum inhibition of *S. rolfssii* up to 57.9% at a concentration of 0.8 mM was observed only in the case of the isoflavan I_3 . The isoflavan H_3 stimulated mycelial growth of *S. rolfssii* at concentrations of 0.05 and 0.2 mM to 23%, while there was no significant activity at a concentration of 0.8 mM.

As in the case of 5,7,4'-trihydroxy-isoflavan A_3 against *R. solani* and *S. rolfssii* in the present investigation, Adesanya *et al.* [12] found that the 7,2',4'-trihydroxy-isoflavan demethylvestitol was fungitoxic to *Cladosporium cucumerinum* and *Aspergillus niger*. However, in our

previous investigations [1, 11] the 6,7,4'-trihydroxy-isoflavan was inactive against different fungi. Also the 6,7,4'-trihydroxy-isoflavone/isoflavanone showed low activity while the corresponding substances of the genistein series A_1 and A_2 were active to some extent. Similar results were obtained by Adesanya *et al.* [12] with genistein. The differences in activity between the 6,7,4'-trihydroxyisoflavone/isoflavanone and the substances A_1 and A_2 may be ascribed to the presence of the hydroxyl group at C-5 in the latter compounds. In other investigations [11, 13, 14], it was found that even a smaller number of hydroxyl groups can confer antifungal activity to isoflavans. This shows that the degree of reduction, the position and the number of hydroxyl groups together constitute important parameters for high activity of the substances.

It was shown earlier [6] that unsubstituted isoflavonoids (isoflavone, isoflavanone, isoflavan) possessed weak antifungal activity and only after introducing suitable substituents in particular positions were these isoflavonoid structures fungitoxic. In our present investigation, we find that the substituent pattern of B_{1-3} series, viz. two hydroxyl groups at C-5 and C-7 and one methoxy group at C-4', is conducive to a high antifungal activity. In this case the degree of reduction seems to play only a minor role.

Among the tested substances, a comparison of the effectiveness of the isoflavones genistein (A_1) and biochanin A (B_1) reveals that the presence of a methoxy group instead of the hydroxyl group at C-4' bestows a high activity. Johnson *et al.* [15] obtained similar results when testing these two substances against *Cercospora beticola* and *Monilia fructicola*. Furthermore, the isoflavone B_1 is even more active than the isoflavan B_3 . This is in contrast to the hypothesis that a skewed, aplanar molecular shape of the isoflavans [16, 17] is essential for a high activity [8]. In one of our investigations [6], it was found that the 6,7-dihydroxy-4'-methoxy-isoflavan was fungitoxic to different moulds of the genus *Aspergillus*. These results led to the conclusion that two hydroxyl



groups and one methoxy group in an isoflavan guarantee a high activity irrespective of the position of the substituents in the molecule, provided one of the rings is disubstituted. This is demonstrated by the isoflavans B_3 , C_3 , D_3 and E_3 , which have two hydroxyl groups and one methoxy group in their molecules and whose ring A is disubstituted. The importance of derivatising phenolic groups to ethers for increased activity was established by Van Etten [16], Ingham [18], Ingham [19] and Matthews and Van Etten [20]. The presence of an ether function apparently protects the substance against microbial transformations [21]. It was also shown [14, 16] that two hydroxyl groups and one substituted oxygen function other than a methyl-ether group make the isoflavan strongly antifungal. Further methylation could impair

the activity. Also in the present investigation, the isoflavans F_3 , G_3 , H_3 and I_3 with two or three methoxy groups were almost inactive. However, sativan, a 7-hydroxy-2',4'-dimethoxy-isoflavan was found to be more active than the corresponding 7,2'-dihydroxy-4'-methoxy-isoflavan vestitol [16]. In a recent investigation [11], it was found that the 6,7-dihydroxy-3'-methyl-isoflavan is highly active.

EXPERIMENTAL

Isoflavonoids. 13 isoflavonoids were tested in 3 concentrations for their effect on mycelial growth of two soil-borne fungi, *R. solani* and *S. rolsii*. Three compounds belong to the 5,7,4'-trihydroxyisoflavone series A_1 – A_3 , while 5,7-dihydroxy-4'-methoxy-isoflavonoids belong to the series B_1 – B_3 . The isoflavonoids of each series differ only in the degree of reduction. Besides the isoflavans A_3 and B_3 seven other isoflavans C_3 – I_3 were also tested (Table 1). In order to study the influence of gradual reduction on the antifungal activity, the isoflavones were catalytically (Pd-C H_2) reduced to isoflavanones and isoflavans. The purity and authenticity of these products were verified by physical methods (NMR and MS). All the substances were synthesized in the Institute of Physiological Chemistry of the University of Bonn.

Fungi and culture. The soil-borne fungi *R. solani* and *S. rolsii* were originally isolated from *Phaseolus vulgaris* and *Iris hollandica* in German soils near Cologne and kept in the fungus collection of the Institut für Pflanzenkrankheiten at Bonn. Both fungi are pathogenic to soybeans [22].

The effect of isoflavonoids on mycelial growth was investigated in liquid culture. The soln contained 30 g malt extract and 3 g peptone per 1 l distilled H_2O . Me_2CO served as the solvent for the addition of isoflavonoids. The solvent concentration in the soln was maintained at 1.1%. Medium (20 ml) including the particular isoflavonoid in the required concentration was transferred to 100 ml Erlenmeyer flasks and inoculated with five small pieces of mycelium (5 mm in diameter). The flasks were incubated at 23–25° on a reciprocal shaker for 7 days.

Concentrations of isoflavonoids. From phytoalexin experiments [1, 23], it is known that concn in the range of 10^{-5} – 10^{-3} M exhibit fungal inhibition. Therefore the concentrations of 0.5, 2.0 and 8.0×10^{-4} M respectively were used in these tests.

Table 1. Effect of isoflavonoids on mycelial growth of *R. solani* and *S. rolsii* (% difference in wt as compared with the control)

Compound	<i>R. solani</i>			<i>S. rolsii</i>		
	0.05 mM	0.2 mM	0.8 mM	0.05 mM	0.2 mM	0.8 mM
A_1		–33.5		–44.5	–64.6	–66.6
A_2	–25.0	–16.8	–50.0	–52.4	–58.7	–63.0
A_3		+31.6	–79.5	+27.7		–91.6
B_1		–50.1	–79.6	–65.4	–57.0	–90.8
B_2		–73.4	–71.1	–26.5	–92.3	–92.1
B_3		–40.5	–76.6		–49.3	–89.2
C_3	–20.6	–79.9	–78.3	–28.8	–90.9	–90.8
D_3	–8.7	–10.1	–86.3		–12.5	–94.3
E_3	–7.9	–16.9	–84.4		–23.7	–93.7
F_3		–31.7	–39.4			
G_3	–29.9	–35.2	–24.2		–36.5	–38.2
H_3	–19.7	–25.7	–23.5	+22.8	+20.6	
I_3	–22.9	–14.6	–15.7	–17.5	–22.9	–57.9

[(+) indicates significant growth stimulation, (–) indicates significant growth inhibition].

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0.8 mM

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-63.0
-91.6
-90.8
-92.1
-89.2
-90.8
-94.3
-93.7

-38.2

-57.9

Evaluations of results. Round filter papers (Schleicher & Schüll No. 595) were weighed after drying for 24 hr at 105°. After filtration of the culture, the residue was dried and weighed as described above. The difference of the weighings gave the dry wt of the fungus. The mean value of eight repetitions for each concentration and fungus was used for calculation. The data were evaluated by analysis of variance. Probability of single differences was calculated at the 5% level.

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ANTIFUNGAL ACTIVITY OF ISOFLAVONOIDS AGAINST STORAGE FUNGI OF THE GENUS *ASPERGILLUS*

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Key Word Index—*Aspergillus*; storage fungi; isoflavonoids; antifungal activity.

Abstract—The fungicidal activity of two isoflavones, one isoflavanone and seven isoflavans was tested in malt extract broth against five storage fungi of the genus *Aspergillus*. While the isoflavones and isoflavanone show only low activity, the two isoflavans 7,8-dihydroxy-4'-methoxyisoflavan and 6,7-dihydroxy-3'-methyloisoflavan were highly inhibitory to *Aspergillus*. Structure-activity relationships are discussed.

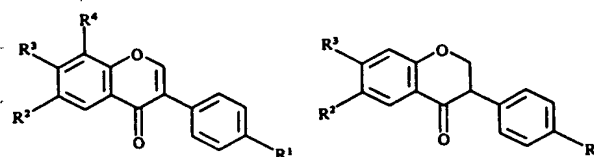
INTRODUCTION

Isoflavonoids are products of secondary plant metabolism of legumes [1] whose antimicrobial effects have been extensively studied [2-7]. The utilization of these natural compounds as substitutes for conventional fungicides in the prevention of plant diseases has been considered [8, 9]. However, to find suitable fungicides in this group of isoflavonoids *in vitro* screenings and the establishment of structure-activity relationships are essential. Such a study may be of immense use in developing some of the isoflavonoids as suitable antifungal substances against storage fungi of the genus *Aspergillus*. The following investigation presents further data in continuation of our previous work in this field [6, 10, 11].

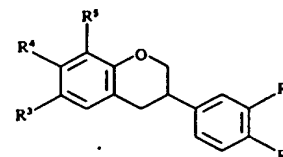
RESULTS AND DISCUSSION

The three isoflavonoids 1-3 showed weak inhibitory activities. Contrary to our previous experience, the isoflavan 3 stimulated the growth of *A. repens* and *A. petrakii*. When compared with 1, the isoflavone 4 is a stronger inhibitor of fungal growth. The isoflavan 5 caused 70.3 and 86.9% growth inhibition of *A. amstelodami* and *A. chevalieri*, respectively. The growth inhibition up to 89.5% of *A. repens* at concentration *c* was statistically not significant. The isoflavan 6 exhibited growth stimulation in the case of the fungi of the *A. glaucus* group (*A. repens*, *A. amstelodami*, *A. chevalieri*) at concentration *a*, but at higher concentrations it was inhibitory. The isoflavan 7 inhibited the growth of *A. petrakii* up to 51.2 and 58.8% at the concentrations *b* and *c*, respectively. The other fungi *A. amstelodami* and *A. chevalieri* were inhibited weakly. The isoflavan 8 was found to be a poor inhibitor of fungal growth. Of all the tested compounds the isoflavan 9 showed the highest activity with all the fungi studied. At concentration *c* it inhibited the growth of fungi up to 98% (excepting *A. petrakii* which was inhibited only up to 73.2%). The isoflavan 10 showed lower inhibitory activity.

Although our previous investigations have demonstrated that isoflavans, in general, possess higher activity



- 1 $R^1 = R^2 = R^3 = OH, R^4 = H$ 2 $R^1 = R^2 = R^3 = OH$
4 $R^1 = OMe, R^2 = H, R^3 = R^4 = OH$



- 3 $R^1 = R^2 = H, R^3 = R^4 = OH$
5 $R^1 = R^3 = H, R^2 = OMe, R^4 = R^5 = OH$
6 $R^1 = R^3 = R^5 = H, R^2 = R^4 = OH$
7 $R^1 = R^2 = R^5 = H, R^3 = OH, R^4 = OMe$
8 $R^1 = R^2 = OMe, R^3 = R^4 = OH, R^5 = H$
9 $R^1 = Me, R^2 = R^3 = H, R^4 = R^5 = OH$
10 $R^1 = R^2 = H, R^3 = OMe, R^4 = R^5 = OCH_3$

than the corresponding isoflavones and isoflavanones [10], all three isoflavonoids 1-3 show a uniform level of low activity. This and other results [7, 12] are contrary to the conclusion that the antifungal activity depends on the skewed, aplanar molecular shape of the isoflavans and pterocarpanes [13]. Meanwhile our own results [11, and unpublished results] and those of Van Etten [7] have been able to show that neither the molecular shape nor the degree of reduction is exclusively responsible for inhibitory activity. The low activity of 6,7,4'-trihydroxyisoflavan (3) [10] as well as of 6,2',4' and 2',4',6'-trihydroxy-2-phenylbenzofuran [14] indicates that several hydroxyl groups in the molecule are not conducive to antifungal activity. Ward *et al.* [15] obtained similar results with trihydroxydihydrophenanthrene. It seems that

the hydroxyl groups contribute to the higher polarity of the molecule which may minimize the fungal membrane permeability of the substances. Smith [16] and Adesanya *et al.* [17] found kievitone to be very effective against different fungal species. This activity was reduced when the more polar kievitone hydrate was examined. Also the oxidation of the lipophilic side chain of kievitone to the hydrophilic primary alcohol resulted in decreased activity. However, 7,2',4'-trihydroxyisoflavan (demethylvestitol) is fungitoxic to *Cladosporium herbarum* and *Aspergillus niger* [17]. It appears that, in addition to the number of polar groups, the position of the substituents in the molecule exerts an influence on the activity. Comparison of the isoflavonoids 4 and 5 with 1-3 reveals that in spite of the common *o*-dihydroxylic groups in both series the presence of a methoxy group in 4 and 5 plays a decisive role in conferring activity. The methoxy group may also be important for the stability of the substances against fungal degradation [18]. While the isoflavan 5 is fungitoxic to *A. glaucus* group with 70-87% inhibition, the isoflavone 4 reduces the growth of *A. repens* at concentration *b* only up to 41%. This corroborates our results that isoflavans with two hydroxyl and one methoxy group are highly active irrespective of the particular substitution pattern [6]. The isoflavan 5 only inhibits *A. flavus* slightly, while there is no effect on mycelial growth of *A. petrakii*. By contrast mycelial growth of *A. glaucus* fungi is strongly inhibited. These results show that fungi vary in their susceptibility to growth inhibition by isoflavonoids [19]. The results obtained with the isoflavan 7 indicate that one hydroxyl and one methoxy group in the molecule are insufficient for high activity [10, 20]. On the other hand, the presence of two hydroxyl groups as in case of the 7,4'-dihydroxyisoflavan 6 produces effective inhibition of the test fungi. O'Neill and Mansfield [20] observed similar effects with 7,2'-dihydroxyisoflavan. However, the antifungal effect of 7,4'-dihydroxyisoflavan against *Aspergillus melleus*, *Penicillium digitatum* and *Fusarium culmorum* is rather low [10]. This shows that two hydroxy groups may guarantee some antifungal activity, but only against certain fungi. In the present investigation, the isoflavan 10 had little effect on mycelial growth of the five species of *Aspergillus*. Considering that the fungi may metabolize isoflavonoids [11, 21-23], a transformation of 10 (demethylenation of the methylenedioxy group) might produce the highly active 6,7-dihydroxy-4'-methoxyisoflavan [6]. However, the genus *Aspergillus* does not appear to be capable of such metabolism and no metabolite of this type could be detected in the extract of the culture medium. The fungicidal effect of the isoflavan 8 is also low, probably due to the presence of methoxy groups at 3'- and 4'-positions [20]. The most effective compound of all is the isoflavan 9. At concentration *c* mycelial growth of all fungi is inhibited up to 70% or more. Whether the methyl group in the B-ring and the two hydroxyl groups in the ring-A of an isoflavan together with the skewed, aplanar molecular shape are responsible for the high activity needs further investigation.

EXPERIMENTAL

The fungi used in this investigation were all soil-borne species of the genus *Aspergillus*: *A. repens* de Bary, *A. amstelodami* (Thom & Church) and *A. chevalieri* (Mangin) Thom & Church of the *Aspergillus glaucus* Link group; *A. flavus* Link of the *Asper-*

Table 1. Effects of isoflavonoids on mycelial growth of five *Aspergillus* species

	<i>Aspergillus repens</i>			<i>Aspergillus amstelodami</i>			<i>Aspergillus chevalieri</i>			<i>Aspergillus flavus</i>			<i>Aspergillus petrakii</i>		
	0.5*	2	8	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8
1															
2				-6.0	-6.6					-13.4	-12.0	-9.5	-18.7	-24.2	-17.8
3	+12.6			-14.0	-15.3		-13.1		-11.3				+8.6		-24.0
4	-19.9	-41.2	-26.5		-25.2	-18.8									+14.5
5						-14.2									
6	+12.5					-25.3									
7				+11.5		-70.3				-14.7	-17.3	-36.8			
8						-54.2	+22.4		-86.9			-10.4			
9	+11.7	-68.7	-97.5	+4.5	-4.2	-14.8	-20.7	-17.8	-67.2				-46.2	-51.2	-39.8
10		-18.3				-98.3			-33.1	+9.9		-23.0			-58.8
									-9.4			-94.5			-73.2

% differences in wt as compared with the control; (+) indicates significant growth stimulation, (-) indicates significant growth inhibition.
*Concentrations are 0.5, 2 and 8 x 10⁻⁴ M/l.

gillus flavus Link group and *A. petrakii* Vörös of the *Aspergillus ochraceus* Wilhelm group. They were isolated from seeds of soybean, pigeon pea, kidney bean, peanut and cotton [24].

The effect of isoflavonoids on mycelial growth was investigated in liquid culture. The solution contained 30 g malt extract and 3 g peptone l dist. H_2O . Me_2CO was used as solvent for the isoflavonoids. The solvent concn in the solution was maintained at 1.1% level. Medium (20 ml) including the particular isoflavonoid in the required concn was transferred to 100 ml flasks and inoculated with five small pieces of mycelium (5 mm in diameter). The flasks were incubated at 23–25° on a reciprocal shaker for 7 days.

Concentrations of isoflavonoids. From phytoalexin experiments [25] it is known that concentrations in the range 10^{-5} – 10^{-3} mol/l exhibit fungal inhibition. Therefore the concentrations of 0.5, 2.0 and 8.0×10^{-4} mol/l denoted as a, b and c respectively were used in these tests.

Evaluations of results. Round filter papers (Schleicher & Schüll No. 595) were weighed after drying for 24 hr at 105°. After filtration of the culture the residue was dried and weighed again. The difference in weights gave the dry wt of the fungi. The mean value of 8 repetitions for each concentration and fungus was used for calculation. The data were evaluated by analysis of variance. Probability of single differences was calculated at the 5% level.

Isoflavonoids. 10 isoflavonoids (Table 1) were tested at 3 concns for their effect on mycelial growth of 5 moulds of the genus *Aspergillus*. These isoflavonoids were obtained by synthesis in the Physiological Chemistry Institute of the University of Bonn, and their purity and authenticity were verified by physical methods (NMR and MS).

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% differences in wt as compared with the control; (+) indicates significant growth stimulation, (–) indicates significant growth inhibition.
*Concentrations are 0.5, 2 and 8×10^{-4} M/l.

–73.2

–94.5

+9.9

–96.1

–33.1

–98.3

–97.5

–18.3

+11.7

10

IN-VITRO VERSUS IN-VIVO ACTIVITIES OF NEW 5-LIPOXYGENASE INHIBITORS WITH ANTIINFLAMMATORY ACTIVITY

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Summary: The possible relationship between in-vitro inhibition of lipoxigenase (LO)/cyclooxygenase (CO) and in-vivo antiinflammatory effects of compounds such as isoflavones (Zy 16369, Zy 16372, Zy 16681) was investigated. The latter were all shown to be potent 5-LO inhibitors when tested in vitro on human peritoneal macrophages ($IC_{50} = 1-7 \mu\text{mol/l}$). Zy 16372 and Zy 16681 also inhibited the 12- and 15-LO and, to a minor extent, the CO. In order to evaluate the antiinflammatory and antiproliferative effects of these compounds in vivo they were applied topically to mice. No definite correlation could be made between the inhibition of the ear oedema induced by arachidonic acid (AA), the inhibition of the epidermal ornithine-decarboxylase (ODC) activity induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), and the in-vitro activities of the compounds. Zy 16372 appeared to inhibit the oedema dose-dependently ($ED_{50} = 5 \mu\text{mol/ear}$) and seemed to be the most potent among the 3 compounds tested and slightly more potent than the reference compound nordihydroguaiaretic acid. As inhibitors of TPA-induced ODC, all 3 compounds exhibited comparable activity. These results suggest that the in-vivo effects of the compounds might be mediated by components other than AA metabolites, and/or be related to their specific kinetic patterns.

Introduction

Metabolites of the arachidonic acid cascade, especially those of the lipoxigenase (LO) pathway, are thought to be involved in the pathogenesis of inflammatory skin diseases such as psoriasis (1,2). Compounds able to block the lipoxigenase activity may represent not only a suitable tool for elucidating the role of some of the mediators involved in inflammatory processes but also a potential therapeutic approach for

inflammatory skin diseases.

Therefore, new isoflavane derivatives were first characterized *in vitro* as lipoxigenase inhibitors in human peritoneal macrophages stimulated with a calcium ionophore. They were then tested *in vivo*, for their anti-inflammatory activity in the arachidonic acid (AA)-induced mouse ear-oedema model (3) and for their antiproliferative activity, by measuring their inhibitory effect on the activity of ornithine decarboxylase (ODC)—induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA)—in the epidermis of hairless mice. The induction of this rate-limiting polyamine biosynthesis enzyme, ODC, might reflect the degree of epidermal hyperplasia (4).

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Materials and methods

Measurement of endogenous eicosanoid production in human peritoneal macrophages

Macrophages were isolated from the ascitic fluid of six patients with alcoholic liver cirrhosis by centrifugation and sedimentation on lymphoprep (Nycomed, Norway). The formation of leukotrienes (LTs), monohydroxy-eicosatetraenoic acids (HETEs) and prostaglandins (PGs) was measured in samples containing 10^6 macrophages per 1 ml Krebs-Henseleit buffer. Macrophages were pre-incubated with the test compounds (0.34, 3.4, 34 and 340 $\mu\text{mol/l}$) for 2 min at 37°C while stirring gently; glutathione (2 mmol/l) was added and after 3 min the cells were stimulated with calcium ionophore A 23187 (2 $\mu\text{mol/l}$). After a further 10-min incubation, the samples were centrifuged for 2 min at $1400 \times g$ and the incubation fluids were kept frozen at -70°C until tested by radioimmunoassay. Determination of LTs, HETEs and PGs was performed in volumes of 10, 25 and 50 μl respectively, with antisera from Advanced Magnetics Inc. (MA, USA). Cross-reactivities were less than 0.2% for related eicosanoids. ^3H -labelled eicosanoids were obtained from Amersham (UK).

Arachidonic acid-induced mouse-ear oedema

Male mice (MAO 1, 25–31 g body weight, 8 animals per group) were anaesthetized and arachidonic acid (Fluka 99% pure, 2 mg/25 μl acetone) was administered to the inner surface of the right ear using a glass micropipette. Test compounds (in 25 μl acetone) were administered topically 40 min prior to arachidonic acid. One hour after arachidonic acid application the mice were killed by cervical dislocation and the thickness of the right and left ears was measured with a micrometer (Mitutoyo). Ear oedema was deter-

mined by subtracting the thickness of the left ear (vehicle-treated ear) from the right ear (compound-treated ear). ED_{50} , defined as the dose of the compound which produces a 50% inhibition of the oedema, was estimated graphically.

ODC activity induced by TPA in the mouse epidermis

Treatment. TPA (Sigma) 10 nmol in 100 μl acetone was applied topically on the right flank over an area of about 10 sq. cm of (HRO)-hairless male mice (20–25 g body weight, 3–5 animals per group) using a glass micropipette. Test compounds (in 100 μl acetone) were administered topically, 40 min prior to TPA application on the same site. All procedures were performed under subdued light. The mice were subsequently housed in the dark until sacrifice in order to minimize photodegradation of the compounds and particularly of all-trans-retinoic acid (RA), used in every experiment as a reference compound. Five hours after TPA application, the mice were killed by cervical dislocation. The skin from each entire treated flank was then cut off, and immediately frozen in dry ice. Skin pieces were kept at -80°C for 1–2 days prior to an ODC assay.

ODC assay. ODC was determined according to the method of M. Connor *et al.* (5), slightly modified in our laboratory. Briefly, the frozen skin pieces were dipped in a 55°C water-bath (0.9% NaCl) for 30 sec, immediately cooled in an ice-cold 0.9% NaCl solution, and then the epidermis was scraped off with a scalpel blade. The epidermis was homogenized at 4°C in a buffer (pH = 7.4) containing 50 mmol/l Tris, 0.1 mmol/l EDTA and 5 mmol/l dithiothreitol (DTT). The homogenate was centrifuged at 4°C for 30 min at $30,000 \times g$. Then, 200 μl of supernatant was removed and pipetted in a reaction vessel containing 200 μl of assay medium (final concentrations of the various reagents in 400 μl total volume

were : Tris 50 mmol/l; EDTA 0.1 mmol/l; DTT 5 mmol/l; pyridoxal phosphate 0.2 mmol/l; L-ornithine 0.1 mmol/l; pH = 7.4) containing 0.25 μ Ci L-(1- C^{14}) ornithine (NEN, 40–60 mCi/mmol).

The reaction vessels were closed and fitted with a filter paper soaked in NCS and incubated at 37°C for 90 min. The reaction was stopped by the addition of 500 μ l of 40% trichloroacetic acid and the $^{14}CO_2$ trapped on the filter paper was evaluated by C^{14} counting in a standard scintillation cocktail. Protein content of the supernatant was determined using the Bio-Rad protein assay. ODC activities are expressed as pmol $^{14}CO_2$ released per mg soluble protein per 90 min.

Results

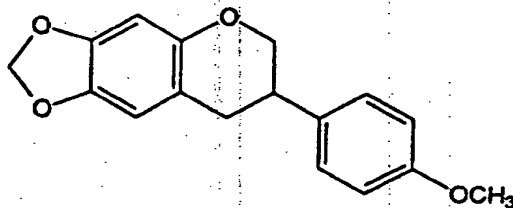
The isoflavane derivatives, the chemical structures of which are presented in Fig. 1, were all found to be 5-LO inhibitors *in vitro* (IC_{50} = 1–5 μ mol/l) in human peritoneal macrophages stimulated by the calcium ionophore A 23187 (Table I). Zy 16372 and Zy 16681 also inhibited

Table I Effects of isoflavane derivatives on eicosanoid production by human peritoneal macrophages.

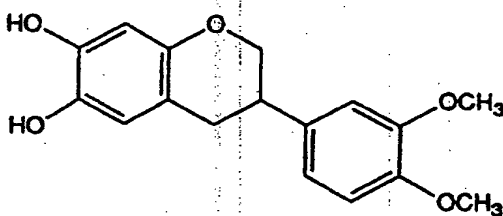
Eicosanoid	Zy 16369	Zy 16372	Zy 16681
	Mean IC_{50} values (μ mol/l)		
LTB ₄	4	5	2
5-HETE	2	2	1
15-HETE	+	9	17
12-HETE	>500	16	20
6kPGF _{1α}	>500	150	140
TxB ₂	20	12	30
PGF _{2α}	n.i.	++	>500
PGD ₂	>500	10	3

+ slightly stimulated; ++ stimulated; n.i. not inhibited.
LT leukotriene, HETE hydroxyeicosatetraenoic acid,
PG prostaglandin, Tx thromboxane.

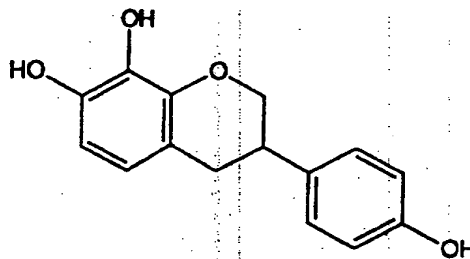
IC_{50} : concentration of the compound yielding a 50% inhibition of mediator release, estimated graphically from dose-response curves.



Zy 16369 (4'-methoxy-6,7-methylenedioxy-isoflavane)



Zy 16372 (6,7-dihydroxy-3',4'-dimethoxy-isoflavane)



Zy 16681 (4',7,8-trihydroxyisoflavane)

Fig. 1 Chemical structures of the isoflavane derivatives tested.

the 12- and 15-LO (IC_{50} = 9–20 μ mol/l). Additionally, the isoflavane derivatives exhibited an inhibitory effect on the formation of some cyclooxygenase products. They were all shown to be thromboxane inhibitors (IC_{50} = 12–30 μ mol/l). Zy 16372 and Zy 16681 inhibited also PGD₂ formation (IC_{50} = 3–10 μ mol/l).

In vivo, in the AA-induced ear oedema (Table II)

Table II Effects of topically applied compounds on arachidonic acid-induced mouse-ear oedema.

Compounds	ED ₅₀ (μ mol/ear)	Maximum inhibition (%) [*] (μ mol/ear)
Test compounds		
Zy 16369	not achieved	5-24 (7)
Zy 16681	not achieved	20-30 (7)
Zy 16372	5	50-55 (5)
Reference compounds		
Nordihydroguaiaretic acid	10	40-50 (10)
Salbutamol	0.004	80 (0.04)
Dexamethasone ^{**}	0.008	70-80 (0.016)

* The increase of ear thickness induced by arachidonic acid alone ranged from 15.4 ± 1.9 to $27.6 \pm 1.9 \times 10^{-2}$ mm (mean \pm S.E.) above control ear thickness depending on the experiment. The maximum inhibition given in the table represents the range of values obtained from several experiments, including dose-response studies.

** Dexamethasone used as a reference compound, was applied 180 min before AA instead of 40 min as for the other compounds.

ED₅₀: dose of the compound yielding a 50% inhibition of the oedema: estimated graphically from dose-response curves.

Zy 16372 seemed to be the most potent among the three isoflavane derivatives tested (ED₅₀ = 5 μ mol/ear). It was slightly more potent than NDGA, the reference compound for LO-inhibitors. However, salbutamol and dexamethasone, used as reference compounds for the test, yielded ED₅₀ values of 4 and 8 nmol/ear respectively.

As inhibitors of TPA-induced ODC (Table III), all three Zy-compounds exhibited comparable activity (max. inhibition: 20-45% at 10-30 μ mol/10 cm²), whereas NDGA did not inhibit the TPA-induced ODC under our experimental conditions. All-trans-retinoic acid, used as reference compound for the test, exhibited an ED₅₀ lower

Table III Effects of topically applied compounds on the phospholipase A₂-induced ornithine decarboxylase (ODC) activity in hairless mouse epidermis.

Compounds	Maximum inhibition (%) [*] (μ mol/10 cm ²)
Test compounds	
Zy 16681	30-40 ^{**} (from 1 to 30)
Zy 16369	20-40 ^{**} (from 1 to 10)
Zy 16372	40-45 (20)
Reference compounds	
Nordihydroguaiaretic acid	n.i. (30)
Retinoic acid (all-trans)	70-90 (0.002)

* Depending on the experiment, basal levels of ODC activity in epidermis ranged from 10.7 ± 4.4 to 21.0 ± 1.4 and TPA-induced ODC activity from 5.588 ± 1.081 to 8.126 ± 1.468 pmol ¹⁴CO₂/mg protein in 90 min incubation (mean \pm S.E.). The maximum inhibition given in the table represents the range of values obtained from several experiments, including dose-response studies.

** Not dose-dependent
n.i.: No inhibition.

than 2 nmol/10 cm².

Discussion

Inflammation and epidermal hyperproliferation are among the features most prominent in several skin diseases. Furthermore, inflammatory processes are thought to involve metabolites of the arachidonic acid cascade.

New isoflavane derivatives were evaluated *in vitro* for their effects on eicosanoid production and found to inhibit the release of LO products and, to a minor extent, CO products. Compared with results obtained from the literature, these isoflavane derivatives exhibited a potency as 5- and 12-LO inhibitors similar to that of the known LO/CO-inhibitors phenidone and NDGA (6).

The compounds were then tested *in vivo* in an inflammatory model (AA-induced mouse ear oedema), involving LO and CO products as main mediators of inflammation (7). When tested on the AA-induced ear oedema model, the reference compounds salbutamol, dexamethasone and NDGA yielded ED₅₀ values in agreement with published data (3, 8). Salbutamol and dexamethasone, which are known to be active by mechanisms other than LO/CO enzyme inhibition, were about 1,000 times more potent than NDGA. Zy 16372, the most active among the isoflavane derivatives tested, and NDGA, the reference compound for LO/CO inhibitors, exhibited a comparable, moderate inhibitory effect on ear oedema.

The isoflavane derivatives were further tested in a model possibly mimicking a pathophysiological epidermal proliferation (TPA-induced ODC). In this latter model, lipoxigenase inhibitors were reported to inhibit the induction of ODC by TPA (9).

Results described in the literature indicate that LO/CO-inhibitors such as NDGA and AA 861, a benzoquinone derivative, inhibited TPA-induced ODC activity by 50% and 80% respectively (10, 11). In our experiments NDGA did not inhibit the TPA-induced ODC. The apparent discrepancy between our results and published data concerning NDGA might be explained by differences in methodology (TPA dosage for ODC induction, strains of mice hairless HRO versus CD-1). In contrast, the isoflavane derivatives were comparably and moderately inhibitory of the induction of ODC activity by TPA.

Although products of the lipoxigenase pathway are thought to be mediators of the ear oedema induced by AA and to be involved in the induction of ODC by TPA, the results that we obtained showed that the isoflavane derivatives tested and NDGA did not exert a potent inhibitory effect in either of the *in-vivo* tests.

A definite correlation between the *in-vitro* and *in-vivo* activities of isoflavane derivatives could not

be ascertained. Our results suggest that both AA-induced oedema and TPA-induced ODC involve more features than the products of the LO/CO pathway alone. Moreover, kinetic parameters specific for the compounds (skin penetration and/or metabolization) might explain the moderate *in-vivo* activities of the compounds.

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Effects of alpha-tocopherol, its carboxylic acid chromane compound and two novel antioxidant isoflavanones on prostaglandin H synthase activity and autodeactivation

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Summary. The natural antioxidant alpha-tocopherol has repeatedly been described to inhibit platelet aggregation and thromboxane formation, whereas its influence on prostaglandin H synthase in vivo and in vitro is a matter of controversy. In the present study the effects of different antioxidative compounds on ram vesicular gland microsomal prostaglandin H synthase activity were investigated in vitro: d,l-alpha-tocopherol, its carboxylic acid chromane compound (Trolox), phytol, alpha-tocopherolacetate and two novel antioxidative isoflavanones, obtained by methylation and/or hydrogenation of naturally occurring isoflavones from fermented soybeans (6,7-dihydroxy-4'-methoxyisoflavanone and 6,7,4'-trihydroxyisoflavanone). Alpha-tocopherol, -acetate and phytol revealed no significant influence on the enzyme activity when applied in concentrations up to 1 mM. Trolox (100–1000 µmol/l) and the two isoflavanones (5–50 and 10–100 µmol/l) dose-dependently augmented the initial rate of oxygen consumption and the total oxygen uptake during prostaglandin H synthase incubation with arachidonic acid (AA). In parallel, these compounds increased the formation of prostaglandin E₂ and F₂ alpha from ¹⁴C-labelled AA, and they markedly protected the prostaglandin H synthase from rapid autodeactivation as revealed by repetitive application of AA in small doses. We suggest that these compounds serve as cosubstrates to which the oxidizing equivalents are transferred which arise during the hydroperoxidase reaction of the enzyme.

Key words: Prostaglandin H synthase – Prostaglandins – Alpha-tocopherol – Isoflavanones – Antioxidant

Introduction

d,l-Alpha-tocopherol has been described to inhibit platelet aggregation and release reaction in vitro and in vivo (Steiner and Anastasi 1976; Steiner 1978; White et al. 1977). Elevated platelet thromboxane and malonaldehyde production in states of vitamin E deficiency (Pritchard et al. 1982; Hamelin and Chan 1983) and a moderate but consistent reduction of platelet prostanoid generation by high dose alpha-toco-

pherol supplementation suggested an inhibitory effect of antioxidative vitamin on platelet prostaglandin H synthase activity (Ali et al. 1980; Gilber et al. 1983; Mower and Steiner 1983). Similarly, serum levels of PGE₂ and PGF₂ alpha revealed an inverse relationship to the serum concentrations of alpha-tocopherol (Hope et al. 1975; MacLennan 1978). In contrast, the activity of the rabbit semitendinous muscle prostaglandin H synthase was found to be decreased during vitamin E deficiency (Chan et al. 1980). In rat renal medulla slices, alpha-tocopherol at a concentration of 5 mmol/l revealed no influence on PGE₂ and PGF₂ alpha generation (Zenser and Davis 1978). Controversial observations were also reported on the vitamin E effect on prostaglandin H synthase in vitro: whereas Nugteren et al. (1978) (absorbance measurement at 278 nm) and Vanderhoek and Lands (1973) (oxygen uptake measurement) described inhibitory effects of high doses of vitamin E, Panganamala et al. (1977, 1982) and Rao et al. (1979) found no influence of the compound on the conversion of ¹⁴C-labelled arachidonic acid (AA) to prostanoids. In the present study we investigated the effects of different antioxidative compounds on ram vesicular gland microsomal prostaglandin H synthase in different test systems in vitro. The experiments included alpha-tocopherol, its carboxylic acid chromane compound (Trolox) as well as two novel antioxidative isoflavanones obtained by hydrogenation of naturally occurring isoflavones from fermented soybeans (so called "Tempeh").

Materials and methods

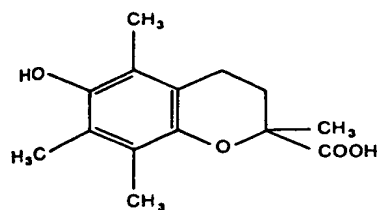
Substances

6,7-Dihydroxy-4'-methoxyisoflavanone and 6,7,4'-trihydroxyisoflavanone (Fig. 1) were generously provided by Prof. Dr. Zilliken (Bonn, FRG). d,l-Alpha-tocopherol and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, Fig. 1) were kindly supplied by Hoffmann-La Roche (Basel, Switzerland) and indomethacin by Merck Sharp & Dohme (Darmstadt, FRG). Arachidonic acid (>99% purity), d,l-alpha-tocopherolacetate and phytol were purchased from Sigma (Munich, FRG). Epinephrine was obtained from Fluka (Neu-Ulm, FRG) and hydrogen peroxide (35%) from Riedel-de Haën, Seelze, FRG). The concentration of the hydrogen peroxide stock solution was controlled repeatedly by means of the glutathione peroxidase assay in the absence of catalase (Seeger et al. 1984). All other reagents were supplied by Merck (Darmstadt, FRG) and used in p.a. quality.

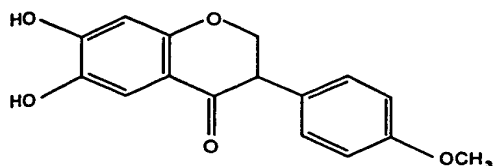
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* This manuscript includes parts of the thesis of U. Moser

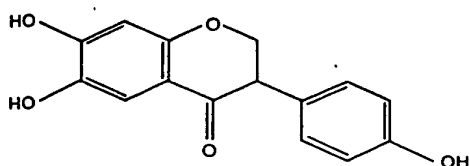
Abbreviations. AA, arachidonic acid; PG, prostaglandin; IC₅₀, concentration for 50% inhibition; v_i, initial rate of oxygen consumption; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; pO₂, oxygen tension in solution



6 - hydroxy - 2,5,7,8 - tetramethylchroman - 2 - carboxylic acid (Trolox)



6,7 - dihydroxy - 4' - methoxyisoflavanone



6,7,4' - trihydroxyisoflavanone

Fig. 1. Structures of Trolox, 6,7-dihydroxy-4'-methoxyisoflavanone and 6,7,4'-trihydroxyisoflavanone

^{14}C -Arachidonic acid (specific activity 53.8 mCi/mmol), ^3H -prostaglandin E_2 (specific activity 150 Ci/mmol) and ^3H -prostaglandin F_2 α (specific activity 145 Ci/mmol) were purchased from Amersham-Buchler (Braunschweig, FRG). ^3H -6-keto PGF_1 α (120–180 Ci/mmol) and ^3H -thromboxane B_2 (100–150 Ci/mmol) were from New England Nuclear (Dreieich, FRG).

Preparation of ram vesicular gland microsomal fraction. The isolation procedure was similar to that described by Nugteren and Hazelhof (1973). The vesicular glands were obtained from a local slaughter house and were cleaned and homogenized with nine volumes (w/v) Tris-HCl buffer (0.1 mol/l, pH 8.3), using an Ultra Turrax homogenizer. The homogenate was sonicated for 2 min at 0°C and centrifuged at $10,000 \times g$ for 20 min at 4°C . The supernatant was filtered through gauze and centrifuged at $105,000 \times g$ for 1 h at 4°C , whereafter the pellet was resuspended in Tris-HCl buffer and again centrifuged at $105,000 \times g$ for 1 h. This pellet was taken up in water, shortly homogenized and lyophilized. The dry microsomal fraction was aliquoted and stored at -70°C until use. The protein content was determined according to Lowry et al. (1951).

Oxygen incorporation measurements. The incorporation of oxygen into AA was determined by measuring the change of oxygen tension in solution ($p\text{O}_2$) according to Egan et al. (1976). $p\text{O}_2$ of the gas-tight oxygen monitor chamber was measured by a Clark oxygen electrode (Nilsson et al. 1981) with a reaction time of <3 s (Eschweiler, FRG). The electrode was regularly calibrated by use of a freshly prepared Na_2SO_3 solution at 37°C (zero) and Tris-HCl buffer solutions (0.1 mol/l pH 8.3, 37°C) which had been equilibrated

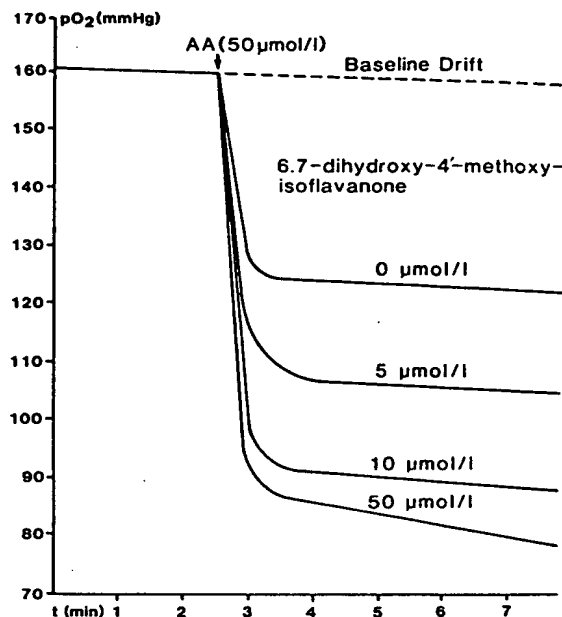


Fig. 2. Examples of the slope of oxygen consumption following admixture of AA to prostaglandin H synthase in the absence and in the presence of different concentrations of 6,7-dihydroxy-4'-methoxyisoflavanone. The baseline drift of $p\text{O}_2$ is indicated

with calibration gases containing 5% O_2 or 15% O_2 . The water-jacketed oxygen monitor chamber, containing 2.3 ml Tris-HCl buffer (0.1 mol/l, pH 8.3) and 0.3 mg microsomal protein, was kept at a constant temperature of 37°C . Compounds to be tested, dissolved in 5 μl ethanol, or an equivalent volume of the vehicle alone were admixed and the oxygen tension was recorded for 2 min. A decrease in $p\text{O}_2$ of <1.5 mm Hg/min was accepted during this phase. The reaction was then initiated by addition of AA in 5 μl ethanol to give a final concentration of 50 $\mu\text{mol/l}$. Initial reaction rates (v_i) were determined from the linear portion of the oxygen monitor trace at the onset of the reaction (Fig. 2). Additionally, the oxygen consumption after 5 min was read, corrected for the baseline drift noted in the 2-min-preincubation period. Oxygen tension was converted to oxygen concentration in solution according to the Charles law (on the basis of a solubility coefficient of O_2 in water of $0.0234 \text{ cm}^3/\text{g H}_2\text{O}$ (37°C , 760 mm Hg) and the Boyle Mariotte gas law, 1.21 $\mu\text{mol/l O}_2$ is calculated for an oxygen tension of 1 mm Hg). In separate experiments it was checked that none of the compounds to be tested initiated a decrease in $p\text{O}_2$ in the absence of the microsomal protein that surpassed the allowed $p\text{O}_2$ baseline drift of 1.5 mm Hg/min.

Radiochromatographic measurement of ^{14}C -AA oxygenation. The reaction conditions corresponded to those during the oxygen incorporation measurement. The reaction chamber (37°C) contained 2.3 ml Tris-HCl buffer (0.1 mol/l, pH 8.3) and 0.3 mg microsomal protein. Compounds to be tested in 5 μl ethanol or the vehicle alone were admixed for 2 min and the reaction was started by addition of unlabelled AA and 30,000 dpm ^{14}C -AA in 5 μl ethanol to give a final concentration of 50 $\mu\text{mol/l}$. After 5 min the reaction was terminated by addition of 25 mg SnCl_2 in 5 ml ethanol, thereby converting the endoperoxides synthesized preferentially to

PGE₂ and PGE₂ α (Yoshimoto et al. 1977; Hemler et al. 1976). The whole volume was acidified to pH 3.5 by addition of citric acid (2 mol/l) and was extracted twice with 4.5 ml ethyl acetate. The solvent was evaporated, the residue redissolved in 100 μ l ethyl acetate and 20 μ l were spotted on the concentration zone of a silica gel plate (60 F₂₅₄; Merck, Darmstadt, FRG) under nitrogen-stream. Labelled standards were spotted on the same plate, and the chromatogram was developed under N₂ three times, using ethyl acetate — glacial acetic acid 99:1 as mobile phase. The pattern of radioactivity on the plates was determined qualitatively with a Berthold radiochromatogram scanner and peaks were designated by comparison to the standards. The whole silica gel was scraped off in fractions and radioactive material was extracted by shaking vigorously for 2 min with 10 ml scintillation fluid. Radioactivity was measured with a Beckman liquid scintillation spectrometer. Counting efficiency was determined by external standardization. All results were calculated as desintegrations per min and all determinations were performed twice. Radioactivity corresponding to the PGE₂ and PGF₂ α peaks were given relative to the total radioactivity on the plates.

Photometric measurement of the hydroperoxidase-activity of prostaglandin H synthase. The hydroperoxidase-activity was measured as described (Takeguchi and Shi 1972; Rahimtula and O'Brien 1976). The cuvette at 37°C contained 1 mmol/l EDTA, 1 mmol/l epinephrine and 50 μ g microsomal protein in 1 ml Tris-HCl buffer (0.5 mol/l, pH 9.0). Compounds to be tested in 5 μ l ethanol or the vehicle alone were incubated for 1 min. The reaction was started by admixture of 400 μ mol/l H₂O₂ and the formation of adrenochrome was recorded at 492 nm. The initial (linear) increase in optical density in the presence of the compound to be tested ($\Delta E/\text{min}_c$) was given relative to the $\Delta E/\text{min}$ in the presence of the vehicle only ($\Delta E/\text{min}_v$) according to:

$$\Delta E/\text{min}_c / \Delta E/\text{min}_v \times 100 (\%).$$

In the given concentration, the vehicle had virtually no influence. The background adrenochrome formation in the absence of H₂O₂ or in the absence of the microsomal fraction never exceeded 5% of the optical density increase of the standard reaction, except for 6,7-dihydroxy-4'-methoxyisoflavanone. This compound initiated a rapid adrenochrome formation when mixed with epinephrine alone and was therefore not used in this assay.

Results

Oxygen incorporation measurements

In the standard reaction mixture, containing microsomal protein and AA, oxygen was consumed with an initial rate of $89.1 \pm 10.3 \mu\text{mol}/\text{min}$ (mean \pm SD, $n = 14$) (Fig. 2). Within <30 s the slope of oxygen consumption declined and the total oxygen uptake measured after 5 min was $51.2 \pm 6.5 \mu\text{mol}/\text{l}$. Considering the stoichiometry proportions of the prostaglandin H synthase step, in the course of which 2 mol of O₂ are incorporated per mol of AA, about half of the substrate was metabolized according to this method after 5 min. Indomethacin dose-dependently inhibited both the initial velocity of oxygen consumption and the total oxygen uptake measured after 5 min with an IC₅₀ of about 0.05 $\mu\text{mol}/\text{l}$; and at 1 $\mu\text{mol}/\text{l}$ both parameters

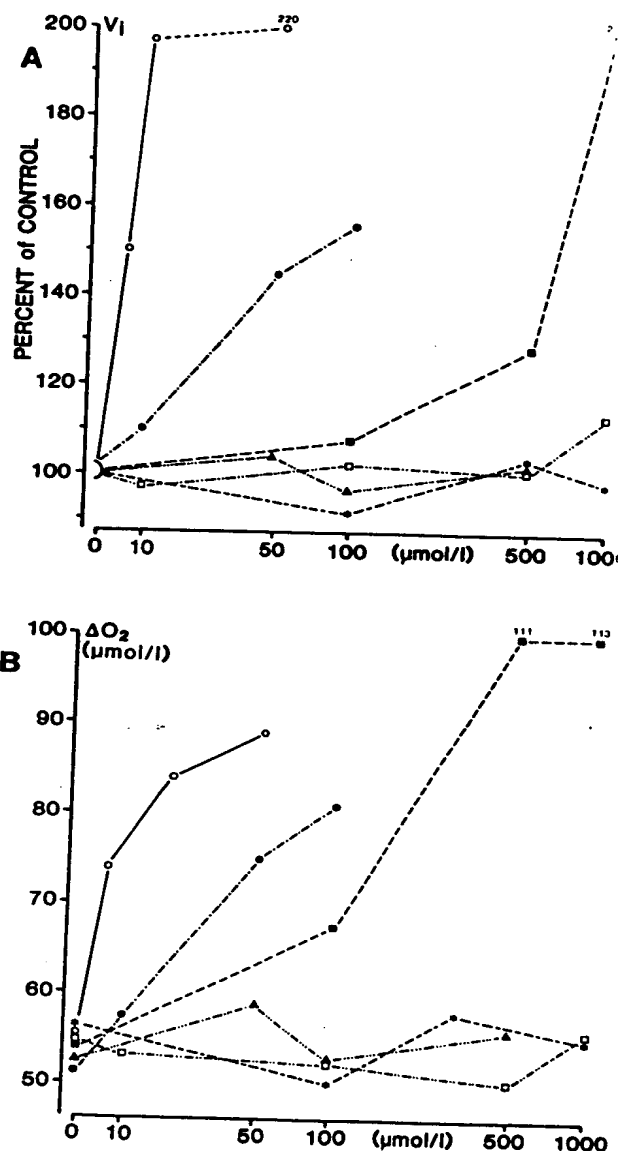


Fig. 3. Dose-effect curves of the different compounds on the initial rate of oxygen consumption (v_i) and the total amount of oxygen uptake after 5 min (ΔO_2). A v_i in presence of the different compounds is given in percent of the initial velocity of O₂ uptake of a standard experiment with vehicle only that was performed in parallel. B ΔO_2 is given as decrease of soluble oxygen ($\mu\text{mol}/\text{l}$) in presence of the different compounds. The starting point on the y-axis gives the O₂ uptake of a standard experiment with vehicle only that was performed in parallel. Each point is the mean of duplicate experiments. ○—○ 6,7-dihydroxy-4'-methoxyisoflavanone; ●—● 6,7,4-trihydroxyisoflavanone; □—□ α -tocopherol; ■—■ Trolox; ▲—▲ tocopherolacetate; *—* Phytol.

ranged below 10% (experiments performed in duplicate). d,l- α -tocopherol, d,l- α -tocopherolacetate and phytol up to concentrations of 1 mmol/l showed no marked influence on the initial velocity of oxygen uptake nor on the total amount of oxygen consumption (Figs. 3a and b). In contrast, Trolox in concentrations between 100 and 1000 $\mu\text{mol}/\text{l}$ and 6,7-dihydroxy-4'-methoxyisoflavanone be-

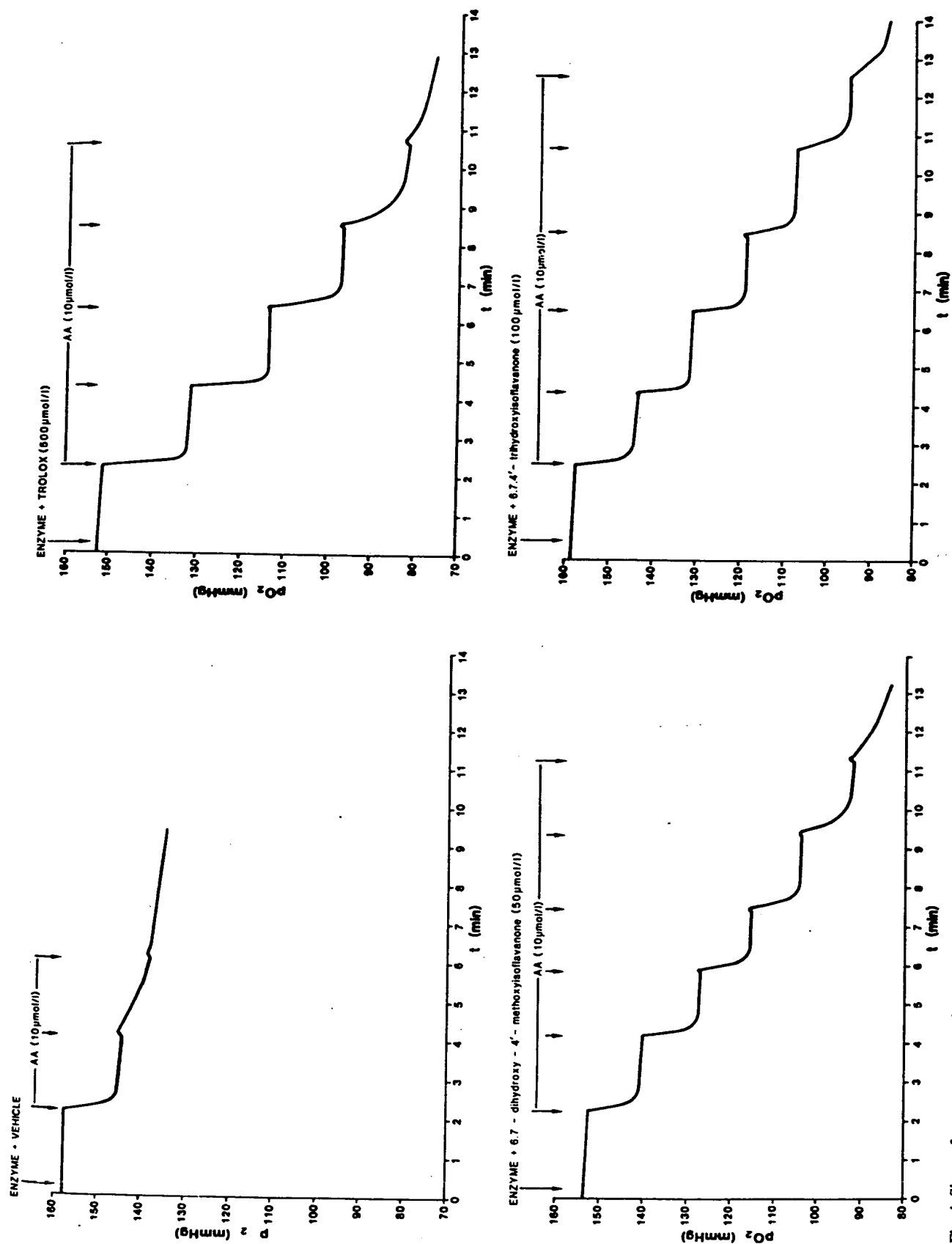


Fig. 4. Slope of oxygen consumption following repetitive application of 10 $\mu\text{mol/l}$ AA (injected in 5 μl ethanol) in absence and presence of different antioxidant compounds. The figure shows one representative experiment out of three each

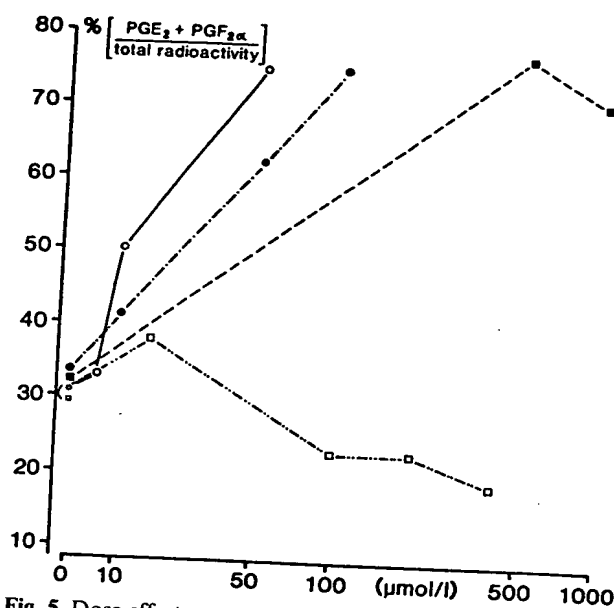


Fig. 5. Dose-effect curves of the different compounds on the conversion of ^{14}C -AA to PGE_2 and $\text{PGF}_2\alpha$. The starting point on the x-axis gives the AA conversion to the two prostanoids of a standard experiment with vehicle only that was performed in parallel. Each point is the mean of duplicate experiments. ○—○ 6,7-dihydroxy-4'-methoxyisoflavanone; ●---● 6,7,4'-trihydroxyisoflavanone; □----□ α -tocopherol; ■-.-.-■ Trolox

tween 5 and 50 $\mu\text{mol/l}$ markedly augmented both parameters (Fig. 3): the initial velocity of oxygen consumption was more than doubled, while the total uptake of O_2 measured after 5 min was about 90 $\mu\text{mol/l}$ for the isoflavanone and 110 $\mu\text{mol/l}$ for Trolox. 6,7,4'-Trihydroxy-isoflavanone induced similar alterations respecting the slope of oxygen uptake, with an increase of both parameters to about 160% of the standard reaction at a concentration of 100 $\mu\text{mol/l}$.

In a separate set of experiments doses of 10 $\mu\text{mol/l}$ AA were repeatedly applied every 2–3 min. In the absence of Trolox or isoflavanone compounds, a rapid O_2 consumption was induced only by the first AA injection (Fig. 4). This feature is known to be caused by the autodeactivation of the enzyme during AA oxygenation (Gale and Egan 1984). In the presence of 50 $\mu\text{mol/l}$ 6,7-dihydroxy-4'-methoxyisoflavanone, of 100 $\mu\text{mol/l}$ 6,7,4'-trihydroxy-isoflavanone or of 500 $\mu\text{mol/l}$ Trolox, the AA injections were followed by a prompt decrease in $p\text{O}_2$ 5–6 times, with a gradual reduction of the initial velocity of oxygen consumption.

Radiochromatographic measurement of ^{14}C -AA oxygenation

In the standard reaction, 30–35% of the total ^{14}C -AA was found to be converted to PGE_2 and $\text{PGF}_2\alpha$ (Fig. 5). This conversion was dose-dependently inhibited by indomethacin, with an IC_{50} of about 0.05 $\mu\text{mol/l}$ (experiments performed in duplicate). Tocopherol slightly suppressed the formation of the two prostaglandins in concentrations between 100 and 400 $\mu\text{mol/l}$. In contrast, Trolox and the two isoflavanones, used in the same concentrations as for the measurement of oxygen consumption, all augmented the percentage of PGE_2 and $\text{PGF}_2\alpha$ formation to 75–80% of the total radioactivity.

Photometric measurement of the hydroperoxidase-activity of prostaglandin H synthase

Up to 500 $\mu\text{mol/l}$ indomethacin did not inhibit this automatic step. Alpha-tocopherol, -acetate and phytol concentrations of 200 $\mu\text{mol/l}$ revealed no significance. Higher concentrations could not be tested because of increasing turbidity caused by these compounds. This caused an increase in the velocity of adrenocortical formation to 133% and 153% in concentrations of 2 mmol/l (experiments in duplicate). Trihydroxyisoflavanone revealed a bidirectional effect with an increase of the rate of absorbance to 143, 145 and 147% at 5, 10 and 50 $\mu\text{mol/l}$ and a decrease to 73, 58 and 48% at 0.1, 1 and 2 mmol/l.

Discussion

During the AA cyclooxygenase step 2 mol of oxygen are consumed per mol of AA metabolized, resulting in the relative slope of the oxygen trace. The initial reaction rate found in the present study (685 nmol O_2/min per mg microsomal protein) corresponds well to that previously described for this assay (600 and 635 nmol/min/mg; Iqbal et al. 1976; Gale and Egan 1984). Oxygen uptake ceases before all the AA or all the oxygen is used because of rapid autodeactivation of the enzyme. This is especially evident in the test system utilizing repetitive application of small doses of AA (Gale and Egan 1984). Calculated from the total oxygen uptake after 5 min, about half of the oxygen was oxygenized in the standard procedure in the present study. Under the same reaction conditions, the radiochromatographic measurement detected a 30–35% conversion of ^{14}C -AA into PGE_2 and $\text{PGF}_2\alpha$. This difference may be explained by the possible formation of additional products: there was radioactivity distinct from the detected PGE_2 and $\text{PGF}_2\alpha$ peaks that was not taken into account by the formula using $\text{PGE}_2 + \text{PGF}_2\alpha/\text{total radioactivity}$. This observation indicates the formation of a small percentage of different AA products contributing to the measured oxygen consumption. Furthermore, there might be some increased non-enzymatic oxygen consumption due to autooxidation of microsomal lipids in the presence of the AA cyclooxygenation process, though there was only a slight O_2 -baseline drift preceding the initial addition of AA. In general, however, the total oxygen uptake after 5 min in the prostaglandin formation from ^{14}C -AA showed consistent responses to the application of indomethacin (inhibition with $\text{IC}_{50} = 0.05 \mu\text{mol/l}$) or the application of the different antioxidative compounds.

Trolox, the chromane compound of alpha-tocopherol bearing only a carboxylic acid side chain, dose-dependently increased the prostaglandin H synthase activity and markedly protected it from rapid autodeactivation in a concentration range between 100 and 1000 $\mu\text{mol/l}$. This compound possesses the same radical scavenging properties as alpha-tocopherol, with the possibility of quinoid redox function (Nishikimi and Machlin 1975; Gallo-Torres 1980; Machlin 1980; Niki et al. 1985; McCay et al. 1982). In general, there may be two sites of interaction of radical scavengers with the prostaglandin H synthase, as this protein possesses two distinct enzymatic properties: the lipoygenase-type of reaction converts AA to the hydroperoxy endoperoxide PGG_2 , while the hydroperoxidase-type reduces the hydroperoxy

group of PGG₂ to the corresponding alcohol PGH₂ (Gale and Egan 1984; Kalyanaraman and Sivarajah 1984; Lands et al. 1984). Radical scavengers may interact with the carbon-centered AA free radical or with AA peroxy radicals arising during the lipoxygenase step (Schreiber et al. 1986). Hydroperoxides are stated as necessary for the activation of this step, and scavenging of the propagating AA peroxy radicals would accordingly result in an inhibition of the prostaglandin H synthase (Lands et al. 1985; Hemler et al. 1979; Hemler and Lands 1980; Taylor et al. 1983). The activating effect of Trolox on the enzyme, however, alternatively suggests that this compound serves as a reducing cosubstrate that is oxidized in conjunction with the hydroperoxidase step. The mechanisms by which oxidizing equivalents are transferred to the oxidizable cosubstrate vary among the substrates and may include both non-radical and radical mechanisms, e.g. release of a soluble species that possesses many characteristics akin to those of the hydroxy radical (O'Brien and Hawco 1978; Egan et al. 1978, 1979, 1981; Kuehl et al. 1980; Marnett 1984; Eling et al. 1985; Markey et al. 1987). As these oxidizing equivalents are supposed to be responsible for the irreversible inactivation of the enzyme, the suggested interaction of Trolox with the hydroperoxidase step is in accordance with the finding of a marked protection against the rapid autodeactivation upon repetitive application of small doses of AA. It is also compatible with the stimulation of adrenochrome formation, when hydrogen peroxide is offered as substrate for the hydroperoxidase step. This view is supported by recent studies by Markey et al. (1987), who noted a direct correlation between the ability of different phenolic compounds to protect against hydroperoxide-induced inactivation of the prostaglandin H synthase and their ability to serve as reducing cosubstrates during the hydroperoxidase step. The higher doses of Trolox needed in the hydroperoxidase assay (1–2 mmol/l) must probably be ascribed to the presence of 1 mmol/l epinephrine, which itself causes activation of the prostaglandin H synthase by serving as a cosubstrate (Kalyanaraman and Sivarajah 1984; Pace-Asciak 1972).

In contrast to its carboxylic acid chromane compound, alpha-tocopherol did not activate the prostaglandin H synthase. This is in accordance to the finding that this vitamin, though being an excellent radical trap, cannot act as a protective agent against the autodeactivation of the enzyme (Porter et al. 1980). There was also no inhibition of the oxygen consumption during AA cyclooxygenation, which is in contrast to the study of Vanderhoek and Lands (1973), describing an inhibitory effect of the vitamin on this parameter with an IC₅₀ of 500 µmol/l. However, their assay system contained 670 µmol/l phenol and therefore additional interactions might be responsible for the difference. This is suggested by a study of Rao et al. (1979), who found no significant effect of tocopherol or nitroblue tetrazolium on the AA conversion by ram vesicular gland microsomes, but an effective inhibition when these agents were applied in combination. The obvious contrast between Trolox, serving as cosubstrate for the prostaglandin H hydroperoxidase reaction with protection against autodeactivation of the enzyme, and the ineffectiveness of the whole vitamin is surprising. The additional phytyl side chain of the vitamin is normally believed to facilitate the access of the antioxidative chromane compound to lipid-rich compartments (Machlin 1980; McCay et al. 1982; Murphy and Davis 1981), and no hindrance of the properties of the chromane compound has

hitherto been described. Adverse effects of the phytyl chain were excluded by the ineffectiveness of phytol itself and by the ineffectiveness of tocopherolacetate, incapable of undergoing redox reactions.

The characteristics of Trolox were mimicked, even in far lower concentrations, by two isoflavanones, possessing a 6-hydroxy group corresponding to the free hydroxy group in the chromane ring of alpha-tocopherol (Fig. 1). These two compounds are methylated and/or reduced derivatives of the 6,7,4'-trihydroxyisoflavone first isolated from "Tempeh" (György 1964), which is prepared by the fermentation of boiled soybeans through fungal activity (*Rhizopus oryzae*) and which is consumed regularly in Indonesian countries. The antioxidative compound in Tempeh is obviously either formed de novo or liberated during fermentation from complex, originally inactive compounds, present in unfermented beans. The isoflavanones used in the present study are potent antioxidants in vitro (Zilliken 1981), and they are evidently effective cosubstrates of the prostaglandin H synthase, as deduced from the oxygen consumption- and the ¹⁴C-AA conversion-measurements as well as from the protective effects against the rapid autodeactivation of the enzyme. The direct measurement of the hydroperoxidase step by offering hydrogen peroxide as substrate was not applicable in the case of the 4-methoxy compound, which interacted directly with epinephrine. The bidirectional influence of the trihydroxyisoflavanone in this assay suggests a competition with the indicator epinephrine as cosubstrate for the hydroperoxidase step in higher isoflavanone concentrations, which superimposes the protective effect against the prostaglandin H synthase autodeactivation.

Up to now a variety of flavanoids and chemically related phenolic compounds have been tested for their influence on prostaglandin H synthase (Arturson and Jonsson 1975; Baumann et al. 1979, 1980; Dewhirst 1980; Sekiya and Okuda 1982; Yoshimoto et al. 1983; Alcaraz and Hoult 1985; Markey et al. 1987). Some stimulate the enzyme (e.g. rutin, hypolaetin-8-glucoside, dihydrophenyl-carbonic acid), some inhibit the enzyme (e.g. cyanidanol, luteolin, baicalein, cirsiolol) and some have no effect. The concentrations of 5 and 10 µmol/l of the isoflavanones, noted to be sufficient for marked enzyme activation in the present study, are the lowest hitherto described. The pharmacological profile of Trolox and of the two isoflavanones may be relevant for the evaluation of inflammatory potencies of oxygen centered radicals arising during AA oxygenation (Kuehl et al. 1977, 1980; Hirafuji and Ogura 1985) in the absence of inhibition of prostaglandin formation and associated side-effects and for strategies addressing a protection of prostaglandin H synthase from autoinactivation (Markey et al. 1987).

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Rijksuniv.

FUNGIZIDE WIRKUNG VON ISOFLAVONOIDEN AUF SCHIMMELPILZE DER GATTUNG ASPERGILLUS

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Zusammenfassung

In Entwicklungsländern der Tropen verursachen Lagerpilze Verluste bis zu 30% der jährlich eingelagerten Ernte. Der Einsatz systemischer und nicht-systemischer Mittel in der Lagerhaltung ist mit Problemen verbunden. Eine Alternative wäre der Einsatz von Naturstoffen z.B. Isoflavonoide, die eine hohe antimikrobielle Wirkung besitzen können. In Malzextrakt-Flüssigkulturen wurde in drei Konzentrationen 0,5, 2 und 8×10^{-4} mol/l die Wirksamkeit einiger Isoflavonoide auf das Myzelwachstum von A. repens de Bary, A. amstelodami (Mangin) Thom & Church, A. chevalieri (Mangin) Thom & Church (Aspergillus glaucus Link Gruppe), A. flavus Link (Aspergillus flavus Link Gruppe) und A. petrakii Vöröš (Aspergillus ochraceus Wilhelm Gruppe) getestet. Von den drei unsubstituierten Isoflavonoiden Isoflavon, Isoflavanon und Isoflavan besaß das Isoflavanon mit 52,6% bei A. repens, 57,1% bei A. amstelodami, 37% bei A. chevalieri und 22,3% bei A. petrakii in der Konzentration 2×10^{-4} mol/l und 32,5% bei A. flavus Link in der Konzentration 8×10^{-4} mol/l die beste Wirkung. Um Struktur-Aktivitäts-Beziehungen aufzuklären, wurden auch Isoflavonoide mit OH- und OCH₃-Gruppen getestet. In der Texasin (6, 7 Dihydroxy-4' methoxy-Isoflavon) -Reihe bewirkte das Texasin-Isoflavan die besten Myzelwachstumshemmungen mit 96,9% bei A. repens, 97,9% bei A. amstelodami und 100% bei A. chevalieri in der Konzentration 8×10^{-4} mol/l. Die Wirkung der getesteten Cumestane war verschieden. Das 3', 4' Dihydroxy-Cumestan zeigte keine signifikanten Wirkungen auf das Myzelwachstum. Während das 3', 4' Dihydroxy-5'methoxy-Cumestan mit 20,1% bei A. repens in der Konzentration $0,5 \times 10^{-4}$ mol/l den höchsten Hemmwert in der Cumestan-Reihe erzielte, förderte das 6, 7, 3', 4' Tetrahydroxy-Cumestan das Myzelwachstum von A. flavus bis zu 22,5% in der Konzentration 2×10^{-4} mol/l.

Einleitung

Lagerpilze der Gattungen Aspergillus und Penicillium verursachen vor allem in Ländern der Tropen und Subtropen Verluste bis zu 30% der jährlich eingelagerten Ernte. Eine wirksame Bekämpfung dieser Schimmelpilze könnte die angespannte Ernährungslage in diesen Ländern entscheidend verbessern. Im Vorratsschutz stehen unspezifisch toxisch wirkende systemische und nicht-systemische Fungizide zur Verfügung. Beim Einsatz dieser Stoffe sind Rückstandsprobleme zu erwarten. Eine Alternative ist der Einsatz von Naturstoffen und deren synthetischer Derivate, die schon in geringen Mengen hochwirksam sind. Diese Voraussetzungen erfüllen die Isoflavonoide, Produkte des sekundären Pflanzenstoffwechsels. Wegen ihrer spezifischen und hohen antimikrobiellen Wirkung werden sie bei den Leguminosen als Phytoalexine diskutiert (INGHAM 1984). Ihr natürlicher Charakter schließt Rückstandsprobleme aus.

Material und Methoden

In Flüssigkulturen wurde die Wirksamkeit von neun Isoflavonoiden in drei Konzentrationen auf das Myzelwachstum von fünf Pilzen der Gattung *Aspergillus* getestet. Bei den eingesetzten Substanzen handelt es sich um die unsubstituierten Isoflavonoide und die Isoflavonoide der Texasin (6, 7 Dihydroxy-4' methoxy-Isoflavon) -Reihe. Diese Isoflavonoide unterscheiden sich im Hydrierungsgrad und im Substitutionsmuster. Auch die Cumestane, die sich vom Cumarin-Grundkörper ableiten, werden zu der Gruppe der Isoflavonoide gezählt (Abb. 1).

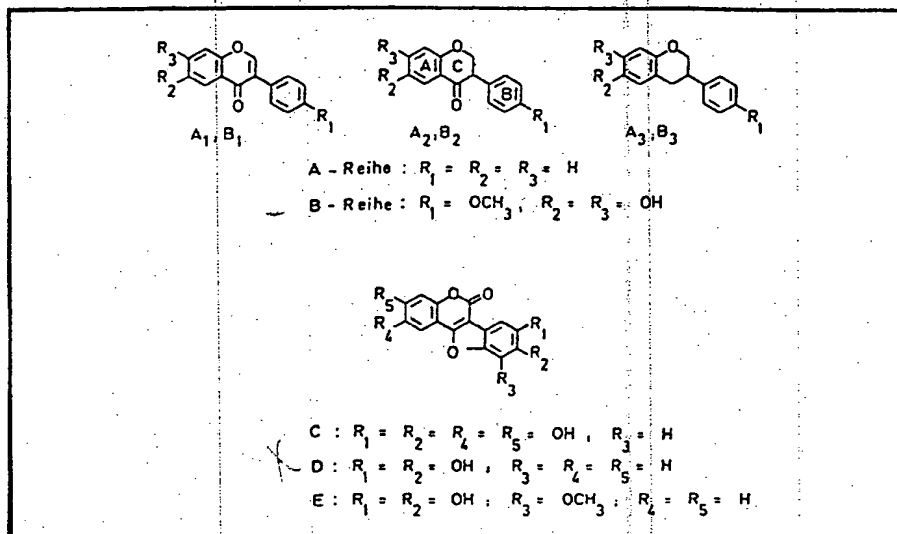


Abb. 1: Strukturformeln der eingesetzten Isoflavonoide

Die in diesen Versuchen verwendeten Pilze zählen zur Klasse der Lagerpilze (CHRISTENSEN und KAUFMANN 1965). *Aspergillus repens* de Bary, *Aspergillus amstelodami* (Mangin) Thom & Church und *Aspergillus chevalieri* (Mangin) Thom & Church sind Vertreter der *Aspergillus glaucus* Link Gruppe. Sie zählen zu den Primärbesiedlern an gelagertem Erntegut. *Aspergillus flavus* Link gehört zur *Aspergillus flavus* Link Gruppe und *Aspergillus petrakii* Vöröš zur *Aspergillus ochraceus* Wilhelm Gruppe. Beide sind gefährliche Toxinbildner.

Malzextraktbouillon wurde bei 1 bar 20 Minuten autoklaviert. In die auf 35°C abgekühlten Nährlösungen wurde die der jeweiligen Konzentration 0,5 , 2 und 8 x 10⁻⁴ mol/l entsprechende in Aceton gelöste Substanzmenge steril eingebracht. Die Acetonkonzentration der Nährlösungen inklusive der Kontrollvarianten betrug 1,1%. In zuvor autoklavierte 100ml Kolben wurden pro Kolben 5 Impfstücke (Durchmesser 5 mm) jeweils eines Pilzes gegeben. Nach Zugabe von 20 ml Nährlösung wurden die Kolben mit Zellulosestopfen verschlossen und eine Woche bei Raumtemperatur auf einem Schüttler belassen. Je Pilz und Konzentration wurden sechs Wiederholungen angesetzt.

Rundfilter (Schleicher & Schüll 90 mm) wurden 24 Stunden bei 105°C getrocknet und dann gewogen. Später wurde der Kolbeninhalt auf die Filter abgesaugt, diese abermals 24 Stunden bei 105°C getrocknet und wiederum gewogen. Aus der Differenz der Werte ergaben sich die Myzel Trockengewichte. Diese Werte wurden dann varianzanalytisch verrechnet.

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Ergebnisse

Das Isoflavon (A_1) zeigte bei *A. amstelodami* mit einer signifikanten Myzelwachstumshemmung von 47,1% in der Konzentration 2×10^{-4} mol/l die stärkste Wirksamkeit, während in der Konzentration 8×10^{-4} mol/l ein Hemmwert von 30,7% erzielt wurde. Bei *A. repens*, *A. chevalieri*, *A. flavus* und *A. petrakii* ergaben sich in der Konzentration 8×10^{-4} mol/l die höchsten Hemmwerte mit 26,1%, 36,1%, 27,2% bzw. 22,5% (Abb. 2).

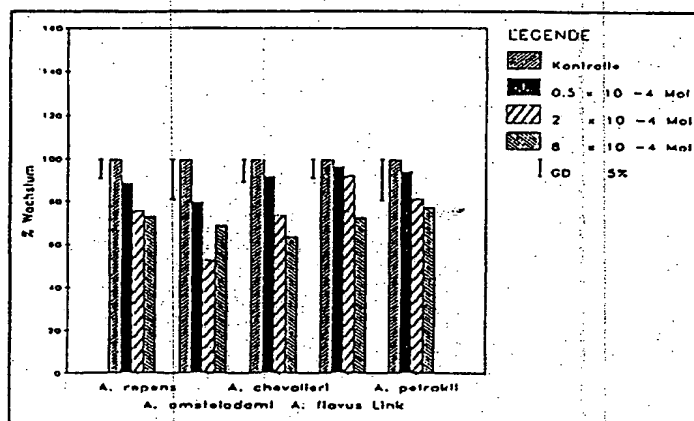


Abb. 2: Myzeltrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von Isoflavon (A_1)

Das Isoflavanon (A_2) bewirkte mit Ausnahme von *A. flavus* in der Konzentration 8×10^{-4} mol/l 32,5% Wachstumshemmung, die höchsten Hemmwerte in der Konzentration 2×10^{-4} mol/l. Das Myzelwachstum von *A. repens* wurde in dieser Konzentration um 52,6%, das von *A. amstelodami* um 57,1%, das von *A. chevalieri* um 37% und das von *A. petrakii* um 22,3% gehemmt. In der Konzentration 8×10^{-4} mol/l konnten bei *A. repens*, *A. amstelodami*, *A. chevalieri* und *A. petrakii* Myzelwachstumshemmungen von 44,8%, 42,7%, 36% bzw. 13,2% erzielt werden (Abb. 3).

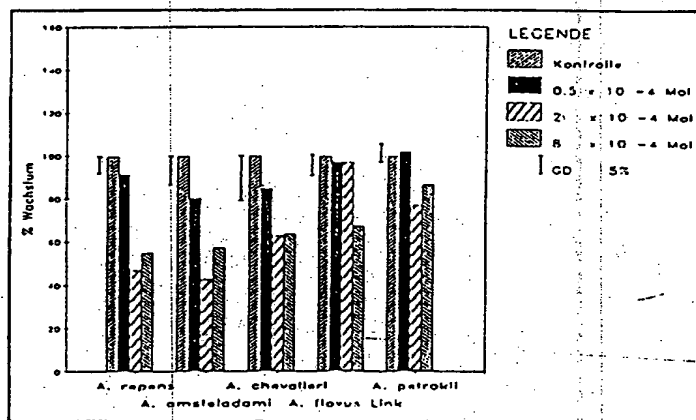


Abb. 3: Myzeltrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von Isoflavanon (A_2)

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Das Isoflavan (A_3) bewirkte nur bei *A. amstelodami* mit 11,8% in der Konzentration 8×10^{-4} mol/l und bei *A. chevallieri* in den Konzentrationen 0,5, 2 und 8×10^{-4} mol/l mit 19,7%, 32,1% bzw. 35,4% signifikante Wachstumshemmungen (Abb. 4).

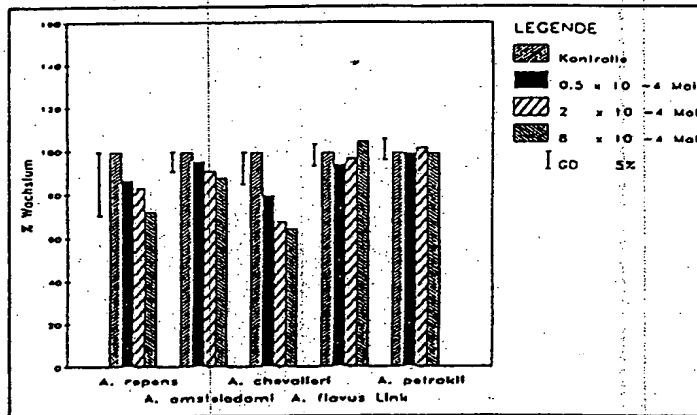


Abb. 4: Myzeltrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von Isoflavan (A_3)

Nur bei zwei Vertretern der *A. glaucus*-Gruppe bewirkte das Texasin-Isoflavan (B_1) signifikante Myzelwachstumshemmungen. Bei *A. repens* betrug der höchste Hemmwert 33,7% in der Konzentration $0,5 \times 10^{-4}$ mol/l, 32% in der Konzentration 2×10^{-4} mol/l und 20,1% in der Konzentration 8×10^{-4} mol/l. *A. amstelodami* wurde in der Konzentration 2×10^{-4} mol/l um 19,6% und in der Konzentration 8×10^{-4} mol/l um 12,1% gehemmt. Dagegen bewirkte das Texasin-Isoflavan (B_1) bei *A. chevallieri* in der Konzentration $0,5 \times 10^{-4}$ mol/l eine signifikante Wachstumsförderung von 11,9% (Abb. 5).

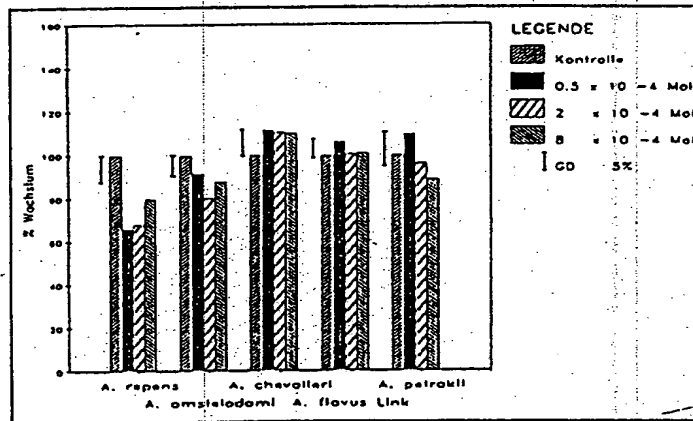


Abb. 5: Myzeltrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von Texasin-Isoflavan (B_1)

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In der Konzentration 2×10^{-4} mol/l erzielte das Texasin-Isoflavanon (B_2) bei allen fünf Pilzen die höchsten Myzelwachstumshemmungen mit 42.3% bei A. repens, 54.9% bei A. amstelodami, 59.5% bei A. chevalieri, 52.3% bei A. flavus und 49.6% bei A. petrakii. Signifikante Inhibierungen des Myzelwachstums in der Konzentration 0.5×10^{-4} mol/l traten nur bei A. amstelodami mit 34.5% und bei A. chevalieri mit 11.9% auf. Das Myzelwachstum von A. amstelodami wurde um 27.7%, das von A. chevalieri um 31.6% und das von A. flavus um 9.6% in der Konzentration 8×10^{-4} mol/l gehemmt (Abb. 6).

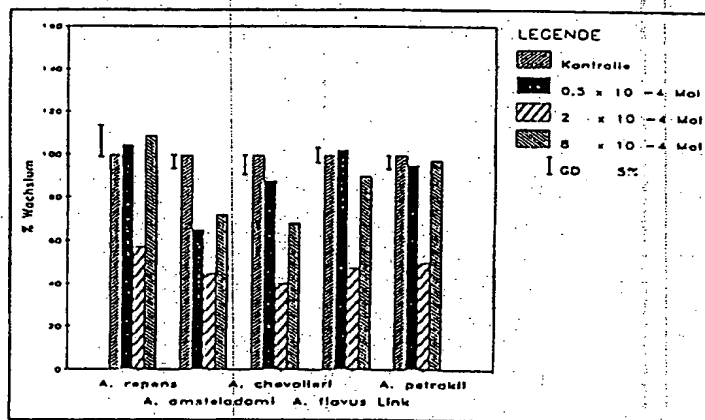


Abb. 6: Myzeltrockenmassebildung von verschiedenen Aspergillus-Arten unter Einfluß verschiedener Konzentrationen von Texasin-Isoflavanon (B_2)

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Die höchsten signifikanten Myzelwachstumshemmungen beim Texasin-Isoflavan (B_2) ergaben sich bei allen fünf Pilzen in der Konzentration 8×10^{-4} mol/l mit 96.9% bei A. repens, 97.9% bei A. amstelodami, 100% bei A. chevalieri, 27.6% bei A. flavus und 44.6% bei A. petrakii. In der Konzentration 2×10^{-4} mol/l trat nur bei A. amstelodami mit 15.6% eine signifikante Wachstumsinhibierung auf (Abb. 7).

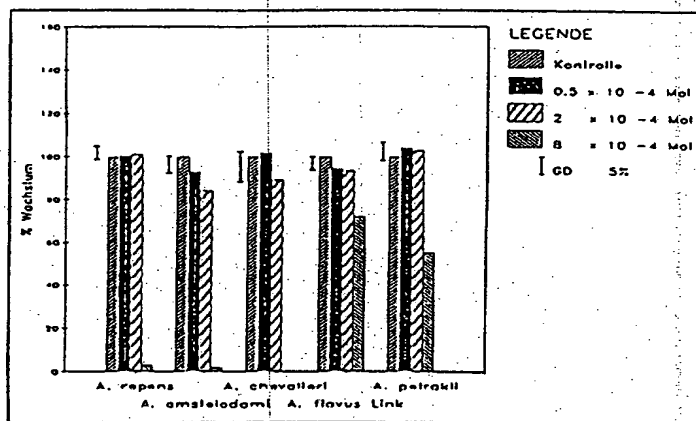


Abb. 7: Myzeltrockenmassebildung von verschiedenen Aspergillus-Arten unter Einfluß verschiedener Konzentrationen von Texasin-Isoflavan (B_2)

influß

Das 6,7,3',4' Tetrahydroxy-Cumestan (C) bewirkte immer eine signifikante Förderung des Myzelwachstums. Diese betrug bei *A. flavus* in der Konzentration $0,5 \times 10^{-4}$ mol/l 18,3%, in der Konzentration 2×10^{-4} mol/l 22,5% und in der Konzentration 8×10^{-4} mol/l 21,1%. Bei *A. petrakii* ergab sich in der Konzentration 2×10^{-4} mol/l eine Wachstumsförderung von 16,4% (Abb. 8).

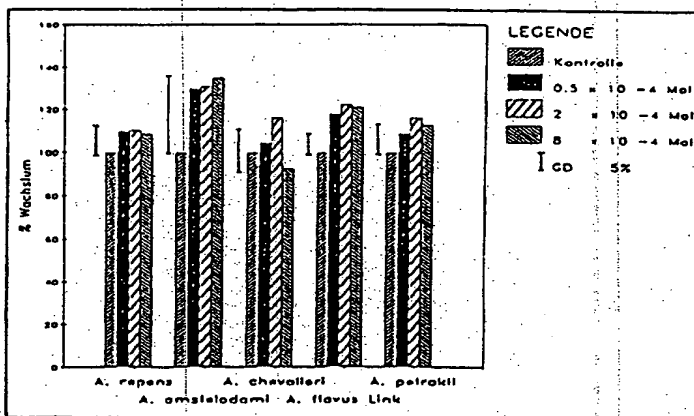


Abb. 8: Myzeltrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von 6,7,3',4' Tetrahydroxy-Cumestan (C)

Weder signifikante Myzelwachstumshemmungen noch Förderungen bewirkte das 3',4'Di-hydroxy-Cumestan (D) bei vier Pilzen. Die Versuchsserie von *A. flavus* war infolge einer Bakterienverunreinigung nicht auswertbar (Abb. 9).

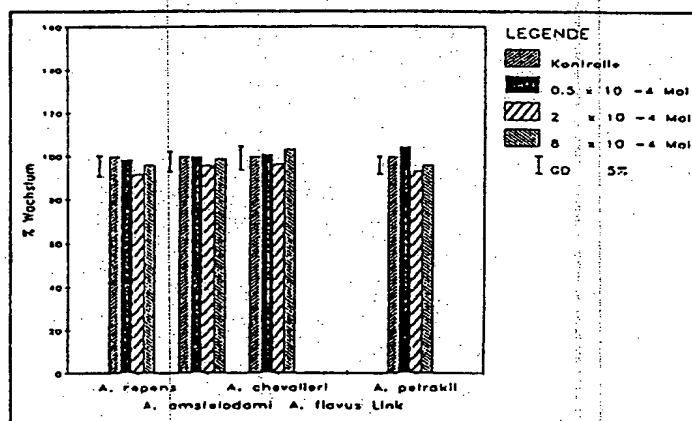


Abb. 9: Myzeltrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von 3',4' Dihydroxy-Cumestan (D)

Leichte signifikante Inhibierungen des Myzelwachstum bewirkte das 3',4' Dihydroxy-5'-methoxy-Cumestan (E) bei *A. repens* mit 20,1% und bei *A. petrakii* mit 9,2% in der Konzentration $0,5 \times 10^{-4}$ mol/l (Abb. 10).

Abb. 10: M

Diskussion

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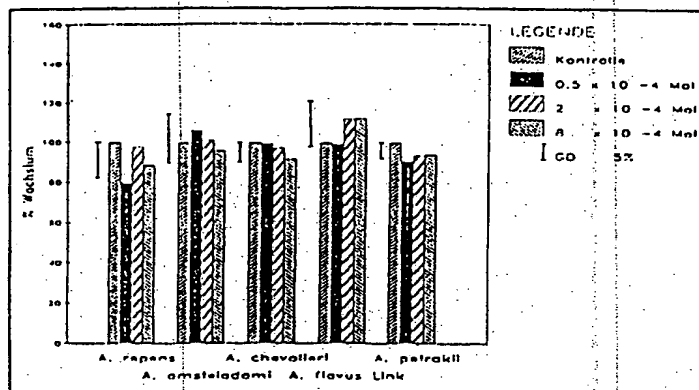


Abb. 10: Myzelrockenmassebildung von verschiedenen *Aspergillus*-Arten unter Einfluß verschiedener Konzentrationen von 3',4'-Dihydroxy-5'-methoxy-Cumestan (E)

Diskussion

Die antimikrobiellen Eigenschaften von Isoflavonoiden sind in einigen Versuchen nachgewiesen worden (CRUICKSHANK 1962, KRAMER et al. 1984). Besondere Beachtung hat die fungizide Wirkung dieser Naturstoffe gefunden. Der Einsatz dieser Stoffe als Ersatz für konventionelle Fungizide wird diskutiert (RATHMELL and SMITH 1980). Persistenz sowie Hydrolyse- und UV-Stabilität dieser Substanzen sind noch weitgehend unerforscht. Eigene Untersuchungen lassen den Schluß zu, daß ihr Einsatz unter Feldbedingungen problematisch sein dürfte. Erfolgversprechender könnte die Verwendung dieser Naturstoffe unter konstanten Umweltbedingungen z.B. im Vorratsschutz sein. Rückstandsprobleme, wie sie beim Einsatz von systemischen und nicht-systemischen Fungiziden entstehen, sind durch den natürlichen Charakter dieser Substanzen ausgeschlossen.

In Flüssigkulturen wurde die fungizide Wirkung von neun Isoflavonoiden auf das Myzelwachstum von fünf Lagerpilzen der Gattung *Aspergillus* untersucht.

Die unsubstituierten Isoflavonoide (A_1 , A_2 , A_3) stellten eine der Versuchsreihen in dieser Untersuchung dar. Die drei Substanzen unterscheiden sich nur im Hydrierungsgrad (Abb. 1). Hierdurch sollte geklärt werden, inwieweit die fungizide Wirkung einer Substanz auf den Isoflavonoid-Grundkörper zurückzuführen ist.

Das Isoflavon (A_1) zeigte bei den drei Vertretern der *A. glaucus*-Gruppe die beste Wirkung. Mit 47.1% Hemmung bei *A. amstelodami* war hier in der Konzentration 2×10^{-4} mol/l der höchste Hemmwert zu verzeichnen. Die Wirksamkeit dieser Substanzen ist also nicht linear konzentrationsabhängig, wie es auch die folgenden Versuchsreihen zeigen. Noch deutlicher zeigt sich diese Tendenz beim Isoflavanon (A_2) mit Ausnahme von *A. flavus*, bei dem mit steigender Konzentration der Substanz auch eine vermehrte Hemmung des Myzelwachstums auftrat. Das Isoflavanon (A_2) erzielte in der Konzentration 2×10^{-4} mol/l immer die höchsten Hemmwerte. Die höchsten Myzelwachstumshemmungen traten auch hier wieder bei den Vertretern der *A. glaucus*-Gruppe auf. Das Isoflavan (A_3) zeigte im Vergleich zum Isoflavon (A_1)/Isoflavanon (A_2) nur eine geringe Wirkung. Dies ist überraschend, da in früheren Flüssigkultur-Versuchen Substanzen mit einem Isoflavan-Grundgerüst aber mit verschiedenen Substituenten Myzelwachstumshemmungen von über 90% bei *Rhizoctonia solani* und *Sclerotium rolfsii* bewirkten. Die reduzierte Wirksamkeit im Vergleich zu den im Ring A und B substituierten Isoflavan-Derivaten ist auf die Abwesenheit der Substituenten zurückzuführen. Eine bessere Metabolisierung durch den Pilz oder schlechtere Durchdringung der Plasmamembran könnten die Ursache für die geringe Wirkung sein (MANSFIELD 1984). Eigene Ergebnisse bestätigen, daß einige Isoflavone/Isoflavanone eine bessere Wirksamkeit als Isoflavane besitzen können. Es scheint, daß die Wirkung der Isoflavonoide spezifisch abhängig vom Pilz, der Substanz und/oder der Konzentration ist. Zu ähnlichen Schlußfolgerungen bei unterschiedlich substituierten Sesquiterpenen kommen auch WARD et al. (1974).

Beim Texasin sind im Ring A an den Positionen 6 und 7 die beiden H-Atome des Isoflavon-Grundkörpers durch jeweils eine OH-Gruppe ersetzt. Im Ring B ist ein H-Atom an der Position 4' durch eine OCH₃-Gruppe substituiert (Abb. 1).

Das Texasin-Isoflavon (B₁) bewirkte nur bei *A. repens* und *A. amstelodami* leichte signifikante Myzelwachstumshemmungen, während bei *A. chevalieri* in der niedrigsten Konzentration eine signifikante Wachstumsförderung auftrat. Keine signifikanten Werte ergaben sich für *A. flavus* und *A. petrakii*. Die reduzierte Wirksamkeit im Vergleich zum Isoflavon (A₁) ist auf den Einbau der beiden OH- und der OCH₃-Gruppe(n) zurückzuführen. Das Texasin-Isoflavanon (B₂) erzielte in der Konzentration 2×10^{-4} mol/l bei allen fünf Pilzen immer die höchste Hemmwirkung. Mit 52.3% bzw. 49.6% konnten in dieser Konzentration bei *A. flavus* bzw. *A. petrakii* die höchsten Hemmwerte überhaupt von allen getesteten Substanzen erzielt werden. Die gute Wirksamkeit vom Texasin-Isoflavanon (B₂) gegenüber dem Isoflavanon (A₂) ist durch die beiden OH- und eine OCH₃-Gruppe(n) bedingt. Im Gegensatz zu allen anderen eingesetzten Substanzen bewirkte das Texasin-Isoflavanon (B₂) eine einheitlich hohe Hemmwirkung in der Konzentration 2×10^{-4} mol/l bei allen fünf Pilzen. Hier zeigt sich nochmal, daß die Wirkung dieser Naturstoffe nicht linear konzentrationsabhängig ist. Auf die Molekülstruktur des Texasin-Isoflavanons (B₂) reagieren auch die nicht Ascosporenbildner *A. flavus* und *A. petrakii* empfindlich. Möglicherweise ist die Metabolisierung für den Pilz erschwert. Auch ein besseres Eindringen durch die Plasmamembran und tiefgreifende Veränderungen im pilzlichen Stoffwechsel können für die gute Wirksamkeit verantwortlich sein. Vom Texasin-Isoflavon (B₁) unterscheidet sich das Texasin-Isoflavanon (B₂) durch den Einbau zweier Wasserstoffatome im Ring C. Diese Reduktion verbesserte die Wirksamkeit stark. Die vollständig hydrierte Variante in dieser Reihe, das Texasin-Isoflavan (B₃), besaß die höchste fungizide Wirkung aller eingesetzten Substanzen. So wurden die drei Vertreter der *A. glaucus*-Gruppe in ihrem Myzelwachstum in der Konzentration 8×10^{-4} mol/l fast vollständig gehemmt. Verglichen mit dem Texasin-Isoflavanon (B₂) war die Wirksamkeit des Texasin-Isoflavans (B₃) bei *A. flavus* geringer; dagegen entsprach die Wirkung des Texasin-Isoflavans (B₃) bei *A. petrakii* der des Texasin-Isoflavanons (B₂) allerdings in der Konzentration 8×10^{-4} mol/l. Die hohe Wirksamkeit von Isoflavanen mit verschiedenen Substituenten ist aus der Literatur bekannt (PERRIN und CRUICKSHANK 1969). Ihre hohe fungizide Wirkung wird auf die aplanare dreidimensionale Molekülstruktur zurückgeführt (VAN ET TEN und PUEPPE 1976). Aus eigenen Versuchen ist aber bekannt, daß nicht nur allein die Molekülstruktur für eine hohe Wirksamkeit verantwortlich ist. Auch die Substituenten sowie deren Anordnung im Molekül beeinflussen die Wirkung. So besitzt das Isoafromosin-Isoflavan mit einer OH-Gruppe an Position 6 und einer OCH₃-Gruppe an Position 7 im Ring A sowie einer OCH₃-Gruppe an Position 4' im Ring B nur eine sehr geringe Wirksamkeit gegen *Rhizoctonia solani* und *Sclerotium rolfsii*. Dagegen bewirkte das Blochanin A-Isoflavan mit zwei OH-Gruppen an Position 5 und 7 im Ring A und einer OCH₃-Gruppe an Position 4' im Ring B sehr hohe Myzelwachstumshemmungen bei diesen beiden Pilzen. Tabelle 1 zeigt, daß die zur *A. glaucus*-Gruppe zählenden Cleistothecienbildner besonders empfindlich auf die unsubstituierten Isoflavonoide und Substanzen der Texasin-Reihe reagieren. Diese Naturstoffe besitzen also eine hohe Wirksamkeit gegen die Hauptfruchtform der Vertreter der *A. glaucus*-Gruppe. Werden keine oder nicht mehr keimfähige Ascosporen gebildet, unterbleibt die Myzelbildung. Dies gilt unter der Voraussetzung, daß bei diesen Pilzen die Myzelbildung ausschließlich durch Ascosporen erfolgt.

Von *A. flavus* und *A. petrakii* ist nur die Nebenfruchtform bekannt. Die Wirkung dieser Substanzen auf die Konidienbildung dieser beiden Pilze ist gering. Eine Ausnahme ist das Texasin-Isoflavanon (B₂) in der Konzentration 2×10^{-4} mol/l und das Texasin-Isoflavan (B₃) in der Konzentration 8×10^{-4} mol/l bei *A. petrakii* (Tabelle 1).

Die Wirkung der drei getesteten Cumestane war verschieden. Während das 6,7,3',4'-Tetrahydroxy-Cumestan (C) bei *A. flavus* in allen drei Konzentrationen das Wachstum signifikant um Ø 20%, bei *A. petrakii* in der Konzentration 2×10^{-4} mol/l um 16.4% förderte, ergaben sich beim 3',4'-Dihydroxy-Cumestan (D) keine signifikanten Werte. Leichte Myzelwachstumshemmungen erzielte das 3',4'-Dihydroxy-5'-methoxy-Cumestan (E) mit 20.1% bei *A. repens* und mit 9.5% bei *A. petrakii* in der Konzentration $0,5 \times 10^{-4}$ mol/l. Aus der Literatur ist bekannt, daß in der Gruppe der Cumestane nur Psoralidin, Cumestrol und Sojagol Phytoalexincharakter besitzen (INGHAM 1984). Mit

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Cumestan
3',4'
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Tab. 1: Signifika
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diesen antimikrobiell wirksamen Stoffen haben die eingesetzten Substanzen nur das Cumarin-Grundgerüst gemeinsam. Sie unterscheiden sich aber von diesen im Substitutionsmuster (Abb.1). Ein Abbau vom 6,7,3',4' Tetrahydroxy-Cumestan (C) ist denkbar, wobei die Metaboliten möglicherweise im Stoffwechsel des Pilzes Verwendung finden. Es wäre auch eine Aktivierung anabolisch wirksamer Enzyme möglich, worauf dann die signifikanten Myzelwachstumsraten zurückzuführen sind. Beim 3',4' Dihydroxy-Cumestan (D) hatte die Substitution zweier H-Atome durch jeweils eine OH-Gruppe weder positiven noch negativen Einfluß auf die Wirksamkeit. Dagegen scheint die OCH₃-Gruppe beim 3',4' Dihydroxy-5' methoxy-Cumestan (E) für die, allerdings geringe, fungizide Wirkung verantwortlich zu sein. Auch beim Pisatin ist die OCH₃-Gruppe für die antimikrobielle Wirkung verantwortlich (MATTHEWS und VAN ETTEN 1983).

	Aspergillus repens			Aspergillus amstelodami			Aspergillus chevalieri			Aspergillus flavus Link			Aspergillus petrakii		
$\times 10^{-4}$ Mol/L	0,5	2	8	0,5	2	8	0,5	2	8	0,5	2	8	0,5	2	8
Isoflavon	-10,3	-23,4	-26,1	-19,9	-47,1	-30,7		-26	-36,1		-27,2				-27,5
Isoflavanon		-52,6	-44,8	-19,5	-57,1	-42,7		-37	-36		-32,5			-22,3	-13,2
Isoflavan						-11,8	-19,7	-32,1	-35,4						
Texasin-Isoflavon	-33,7	-32	-20,1		-19,6	-12,1	-11,9								
Texasin-Isoflavanon		-42,3		-34,5	-54,9	-27,7	-11,9	-59,5	-31,6		-52,3	-9,6		-49,6	
Texasin-Isoflavan			-98,9		-15,6	-97,9		-100			-27,6				-44,6
6,7,3',4' Tetrahydroxy-Cumestan										-18,3	-22,5	-21,1		-16,4	
3',4' Dihydroxy-Cumestan															
3',4' Dihydroxy-5' Methoxy-Cumestan	-20,1													-9,2	

Tab. 1: Signifikante Unterschiede in % in den Myzeltrockengewichten im Vergleich mit der jeweiligen Kontrolle

Die extrem hohe Wirksamkeit vom Texasin-Isoflavan (B₂) gegen die drei Vertreter der A. glaucus-Gruppe ist vielversprechend. Arten der A. glaucus-Gruppe zählen zu den Primärbesiedlern an gelagertem Erntegut. Während ihres Wachstums bilden sie metabolisches Wasser, wodurch die Samenfeuchte erhöht und so die Voraussetzungen für den Befall weiterer Lagerpilze der Gattungen Aspergillus und Penicillium geschaffen werden. Eine wirksame Bekämpfung dieser Lagerpilze mit Naturstoffen könnte die angespannte Ernährungslage in vielen Entwicklungsländern entschärfen. Rückstandsprobleme, die beim Einsatz systemischer und nicht-systemischer Fungizide auftreten, könnten vermieden werden.

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Summary

In tropical developing countries storage fungi cause losses of harvested food grain up to 30% yearly. A control of these moulds with natural substances could be an alternative to non-systemic and systemic fungicides. The isoflavonoids could be taken into consideration as one such group of natural fungicides. The influence of a few selected isoflavonoids was tested in malt extract solution in three concentrations 0.5, 2 and 8 x 10⁻⁴ mol/l on the mycelial growth of five fungi, viz. A. repens de Bary, A. amstelodami (Mangin) Thom & Church and A. chevalieri (Mangin) Thom & Church of the Aspergillus glaucus Link group; A. flavus Link of the Aspergillus flavus Link group and A. petrakii Vörös of the Aspergillus ochraceus Wilhelm group. The isoflavonoids were tested in the series isoflavon, isoflavanon and isoflavan. To establish structure-activity relationship besides unsubstituted isoflavonoids also the methoxy- and hydroxy-isoflavonoids were tested. The isoflavanon caused inhibitions of the mycelial growth up to 57.1% in A. amstelodami at the concentration 2 x 10⁻⁴ mol/l. In the texasin-isoflavonoid (6,7 Dihydroxy-4-methoxy-Isiflavan) series the isoflavan was the most effective one with growth inhibitions of 96.9% in A. repens, 97.9% in A. amstelodami and 100% in A. chevalieri in the concentration 8 x 10⁻⁴ mol/l. Also the coumestans as a further subgroup of natural isoflavonoids were tested in this investigation. The influence of these substances was quite variable. While the 6,7,3',4' tetrahydroxy-coumestan always stimulated the growth (A. flavus 22.5%), the 3',4' dihydroxy-coumestan caused no significant differences in comparison with the control. The 3',4' dihydroxy- 5' methoxy coumestan inhibited the growth of A. repens to 20.1% and the growth of A. petrakii to 9.2% at the concentration 0.5 x 10⁻⁴ mol/l.

Zusammenfas

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PROSTAGLANDINS

6,7,4'-TRIHYDROXYISOFLAVAN : A POTENT AND SELECTIVE INHIBITOR
OF 5-LIPOXYGENASE IN HUMAN AND PORCINE PERIPHERAL BLOOD
LEUKOCYTES

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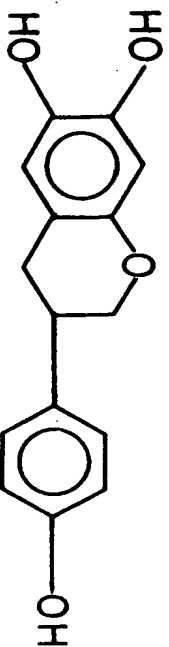
ABSTRACT

The effect of 6,7,4'-trihydroxyisoflavan on human platelet
12-lipoxygenase and human and porcine PMNL 5-lipoxygenase
activities has been studied. 6,7,4'-trihydroxyisoflavan was
found to inhibit 5-lipoxygenase more strongly than 12-lipoxy-
genase ; its concentration for 50 % inhibition (IC_{50}) was
1.6 μM for human and porcine 5-lipoxygenase and 22 μM for
human platelet 12-lipoxygenase. Inhibition of microsomal
cyclooxygenase from ram seminal vesicles is exhibited at
much higher concentrations of 6,7,4'-trihydroxyisoflavan
(IC_{50} = 200 μM).

INTRODUCTION

It is well known, that mammalian lipoxygenases catalyse the
incentive reactions leading to the formation of specific
hydroperoxy derivatives of arachidonic acid. In human
platelets 12-hydroperoxyeicosatetraenoic acid (12-HPETE)
is metabolized in a peroxidase like reaction to 12-hydroxy-
eicosatetraenoic acid (12-HETE). 12-HPETE and 12-HETE are
involved in the regulation of the arachidonic acid meta-
bolism (1,2). 5-Lipoxygenase catalyses specifically the

oxygenation of arachidonic acid at C-5, the initial step in the biosynthesis of 5-HETE and the leukotrienes (LTs) A₄, B₄, C₄, D₄ and E₄. LTC₄, LTD₄ and LTE₄ are responsible for the biological activity of most preparations of the so called "slow-reacting substances" of anaphylaxis (SRS A), possessing great physiological significance in inflammatory processes (3) and hypersensitivity reactions i.e. various allergies (4).



6,7,4'-TRIHYDROXYISOFLAVAN

Fig. 1 : Structure of 6,7,4'-trihydroxyisoflavan

The antioxidative power of various known and novel isoflavonoids, isolated, identified and synthesized in our laboratories, are known and examined since 1975 (5). Recently a remarkable activity of some of these antioxidants has been demonstrated in the recovery of pulmonary vascular resistance as well as the reverse of the increase

in vascular permeability in the perfused isolated shock lung of the rabbit (6). This paper deals with the inhibitory power of 6,7,4'-trihydroxyisoflavan (Fig. 1) on the synthesis of arachidonic acid metabolites in vitro.

METHODS

Materials

Arachidonic acid (E. Merck, Darmstadt) was purified by silicic acid chromatography prior to use. The Ionophore A23187 was obtained from Calbiochem (La Jolla, Cal. USA) (1-¹⁴C)-Arachidonic acid was obtained from New England Nuclear. Dextran T-500 for cell sedimentation was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), prostaglandins and thromboxane B₂ were obtained from Sigma (St. Louis, Mo, USA). PGB₂ was prepared by treatment of PGE₂ with 0.5 M NaOH for 30 min. 5,8,11,14-Eicosatetraynoic acid (ETYA) was supplied by Hoffmann-La Roche, Basle, Switzerland. All organic solvents and pre-coated silica gel plates for TLC were products of E. Merck (Darmstadt, F.R.G.). All chemicals used were of reagent grade.

Preparation of HPETE and HETE standards

12-, 15-, 11-, 9-, 8-, 5-HPETE and the corresponding HETE standards were prepared by air oxidation of arachidonic acid and purified by HPLC according to Porter et al. (7). After methylation, catalytic hydrogenation and trimethylsilylation, the position of the hydroxy moiety was verified by combined liquid gas chromatography-mass spectrometry of the methyl (trimethylsiloxy) eicosanoate derivatives of the HETEs. The derivatives were analysed on a LKB 9000 mass spectrometer. The HETE derivatives due to cleavage

adjacent to the trimethylsiloxy moiety showed the following characteristic fragments: 12-HETE (301, 215 m/e); 15-HETE (343, 173 m/e); 11-HETE (287, 229 m/e); 9-HETE (259, 257 m/e); 8-HETE (245, 271 m/e); and 5-HETE (203, 313 m/e). Each HETE displayed an UV absorption λ_{max} (MeOH) between 235 and 237 nm consistent with a cis, trans-conjugated diene moiety (7).

Preparation of 5S, 12S-DiHETE and LTB₄ standard

5S, 12S-DiHETE was prepared by incubation of porcine leukocytes (100 ml, 30×10^6 cells/ml) stimulated by the ionophore A23187 (10 μM) and treated with ETYA (20 μM) with 12-HETE (5 $\mu\text{g/ml}$) (2). LTB₄ was prepared under similar conditions but with arachidonic acid (100 μM) as the substrate instead (2). After purification by HPLC, the position of the hydroxy moieties was verified by combined liquid gas chromatography-mass spectrometry of the methyl (trimethylsiloxy) eicosanoate derivatives of 5S, 12S-DiHETE and LTB₄. The mass spectra of the two derivatives were identical with the reported mass spectrum of hydroxylated LTB₄ (8). Ultraviolet spectroscopy of the two compounds showed the reported absorption maxima at 258 nm, 268 nm and 278 nm for 5S, 12S-DiHETE (9) and at 260 nm, 270 nm and 281 nm for LTB₄ (10).

Suspensions of peripheral blood leukocytes and platelets

Porcine leukocytes were isolated from peripheral blood collected with one-third volume of 6 % dextran in isotonic saline solution containing 6 units of heparin/ml of blood. The cells were allowed to sediment at 4° C causing the majority of red cells to separate from the white ones. After centrifugation at 500 x g for 20 min, the pellet was resuspended in a HBSS solution containing 0.38%

Na-citrate. The centrifugation was repeated once more. The pellet was resuspended in a Tris-buffered (17 mM, pH 7.2) 0.17 % ammonium chloride solution and incubated at 25° C for 5 min, to induce lysis of the remaining red cells.

After centrifugation at 400 x g for 7 min, the cells were resuspended in phosphate buffered (25 mM, pH 7.4) isotonic saline, 20×10^6 cells/ml. In this cell preparation the mononuclear and polymorphonuclear leukocytes were not separated. The viability of the cells as checked by the trypan blue exclusion test was higher than 90 %. The cell preparations were slightly contaminated with platelets. Human leukocytes were prepared using the same procedure with a slight modification. The first two centrifugations were carried out at 300 x g for 10 min each.

The upper layer of the dextran sedimentation was used for the isolation of the platelets. The supernatant was centrifuged at 1000 x g for 20 min. The pellet was washed two times in phosphate buffered isotonic saline solution.

Conditions of incubation for human and porcine leukocytes and platelets

The cell suspensions were kept at 37° C, then calcium chloride was added to a final concentration of 0.5 mM. Arachidonic acid, ETYA, the ionophore A23187 and 6,7,4'-trihydroxyisoflavan were added in ethanol (the final concentration of ethanol being 0.2 to 0.5 %). For TLC, platelet suspensions were incubated with (1-¹⁴C)-arachidonic acid (55 mCi/mmol, 0.08 μCi totally). For the inhibition experiments cells were preincubated with the inhibitor for 5 min at 37° C. The reaction was initiated by addition of arachidonic acid performed at 37° C for 5 additional min and terminated by the addition of 0.2 volumes of 1 % formic acid and of 2 volumes of chloroform/methanol 1 : 1 (vol/vol).

For quantitative determination of the products in HPLC, prostaglandin B₂ (2 µg) was added as an internal standard.

Extraction, thin layer chromatography and column chromatography

After addition of 2 volumes of chloroform/methanol, the incubation mixtures were immediately cooled in ice, stirred and centrifuged at 500 x g for 10 min at 0°C. The organic layer was withdrawn and evaporated under dry N₂. For TLC the residue was redissolved in a minimal volume of diethylether and spotted on silica thin layer chromatography plates. Plates were developed with either solvent system A, consisting of n-hexane/2-propanol/methanol/acetic acid 65/30/5/0.1 (vol/vol), or solvent system B, consisting of n-hexane/diethylether/methanol/acetic acid 35/50/10/5 (vol/vol). Distribution of the radioactivity on the TLC plates was determined with a Berthold radiochromatogram scanner. Silica gel zones corresponding to TXB₂ and HHT were scraped off and radioactivity was quantitated by a liquid scintillation counter (Packard model 544).

For HPLC the residue was redissolved in 200 µl of the elution solvent and was used for injection 3 times (50 µl at a time). In case of large scale incubations the chloroform extract was purified on a glass column (inside diameter, 0.5 cm) packed with 1 gram of silica gel 60 (Merck) by successive elutions with 30 ml of diethylether/n-hexane, 10/90 (vol/vol), and 30 ml of ethyl acetate. The first eluate contained the unreacted arachidonic acid and other nonpolar lipids. The second eluate contained the mono- and dihydroxy derivatives of arachidonic acid and other polar metabolites.

High pressure liquid chromatography (HPLC)

Analytical HPLC was performed using a prepacked column (Hibar RT 250-4, Lichrosorb 60, 7 µm) from Merck (Darmstadt). The compounds were eluted using first n-hexane/2-propanol/methanol, 973/9/18 (vol/vol), containing 0.1 % acetic acid which was changed after 9.5 min to n-hexane/2-propanol/methanol, 922/60/18 (vol/vol), containing 0.1 % acetic acid. The flow rate was 3 ml/min at 22°C. The elution was monitored by ultraviolet photometry at 235 nm (0 - 9.5 min) and 280 nm (9.5 - 20 min). LTB₄ and 5-HETE were quantitated by comparing the areas of their peaks with that of the internal standard, PGB₂. The extinction coefficients used for PGB₂, LTB₄ and 5-HETE were 26 800, 39 500 and 30 500 respectively.

Partial purification and activity determinations of human platelet 12-lipoxygenase

Human platelet concentrates were obtained from the University blood bank. The platelet concentrate was centrifuged at 300 x g for 15 min to remove erythrocytes. The supernatant was then spun at 2000 x g for 10 min. The platelets were resuspended in 25 mM Tris/HCl buffer (pH 7.5) and lysed by freeze-thawing three times. Preparation of platelet cytosol and precipitation of 12-lipoxygenase was achieved according to the method of Siegel et al. (11). The frozen-thawed homogenate was spun at 4°C for 1 hr at 100 000 x g. The supernatant was saved. The precipitate between 30 and 70 % (NH₄)₂SO₄ saturation was collected by centrifugation at 20 000 x g for 10 min at 4°C. The pellet was dissolved in a minimal volume of 25 mM Tris/HCl at pH 7.5 and was dialysed against the same buffer for 5 hrs. The pH of the dialysed enzyme was adjusted to 6 with 0.1 M HCl and centrifuged again as before. The pH of the supernatant was readjusted to 7.5 with 0.1 M NaOH. This was the enzyme solution for the photometric measurement of human platelet 12-lipoxygenase activity.

12-Lipoxygenase activity was assayed photometrically by the measurement of conjugated diene formation according to Ben-Aziz et al. (12). The 2.5 ml assay mixture for the photometric measurement at 235 nm contained 2.15 ml 0.2 M Tris/HCl buffer pH 7.5 and 0.1 ml enzyme solution. The reaction at 37°C was started after 5 min preincubation at 20°C with or without inhibitor by adding 0.25 ml of substrate solution (0.25 mM arachidonic acid, 0.025 % Tween 20, 0.05 M Na₂HPO₄ pH 9.0). The molar extinction coefficient of 30 500 was used for the determination of enzyme activity.

Preparation of microsomes from ram vesicular glands and determination of cyclooxygenase activity

The isolation procedure has been performed according to the method of Nugteren and Hazelhof (13). Ram vesicular glands obtained from a local slaughter house. The glands were cleaned and homogenized with 3 volume (v/w) of 1 mM EDTA in 0.1 M KH₂PO₄ buffer pH 7.4 using an Ultra Turrax homogenizer. The homogenate was sonicated for 2 min and then filtered through gauze. The filtered homogenate spun at 4000 x g, 4°C, for 10 min. The 4000 x g pellet was discarded and the supernatant was further centrifuged at 105 000 x g for 1 hr. The pellet was taken up in water and lyophilized; yield: 12 g (dry weight) containing 4 - 5 g protein. Incubations were performed according to Ingerowski et al. (14): 0.2 M Tris/HCl, pH 8.0, 1 mM reduced glutathione, 0.05 mM EDTA, 1 mM adrenalin and 6 mg protein from ram vesicular glands preparation in a total volume of 2.0 ml. The reaction, 15 min at 37°C, was started after 5 min preincubation at 20°C with or without inhibitor by adding (1-¹⁴C)-arachidonic acid (0.08 µCi, 55 mCi/nmol) dissolved in 1 % ethanol in 50 mM K₂HPO₄ buffer pH 8.0. Extraction procedure for TLC was the same as described above. For

quantitative determination of cyclooxygenase activity a selective extraction method was used according to Yanagi and Komatsu (15). The unreacted arachidonic acid was extracted with 3 volumes of n-hexane/ethylacetate = 2 : 1 (vol/vol), under neutral conditions. Extraction was repeated twice. The radioactivity in the aqueous phase representing prostaglandin concentrations were measured using a liquid scintillation counter (Packard model 544).

RESULTS

Effect of 6,7,4'-trihydroxyisoflavan upon 5-lipoxygenase derived from porcine and human peripheral blood leukocytes
Incubation of porcine leukocytes with arachidonic acid and ionophore A23187 causes the synthesis of 12-HETE and of 5S, 12S-DiHETE as main products. The presence of ETYA (10 µM) stimulates the synthesis of 5-HETE and LTB₄ and suppresses the formation of 12-HETE and of 5S, 12S-DiHETE. When arachidonic acid and ionophore A23187 were incubated with human leukocytes the main products were 5-HETE and LTB₄. ETYA at the concentration of 10 µM had no effect on ionophore A23187 stimulated 5-lipoxygenase of human leukocytes. Fig. 2A shows a typical HPLC-chromatogram of arachidonic acid metabolites from porcine leukocytes incubated with ionophore A23187 and ETYA. 6,7,4'-Trihydroxyisoflavan inhibits the 5-lipoxygenase completely at a concentration of 15 µM (Fig. 2B). Fig. 3 shows the same inhibitory effect of 6,7,4'-trihydroxyisoflavan on ionophore A23187 activated 5-lipoxygenase from human leukocytes. 6,7,4'-Trihydroxyisoflavan

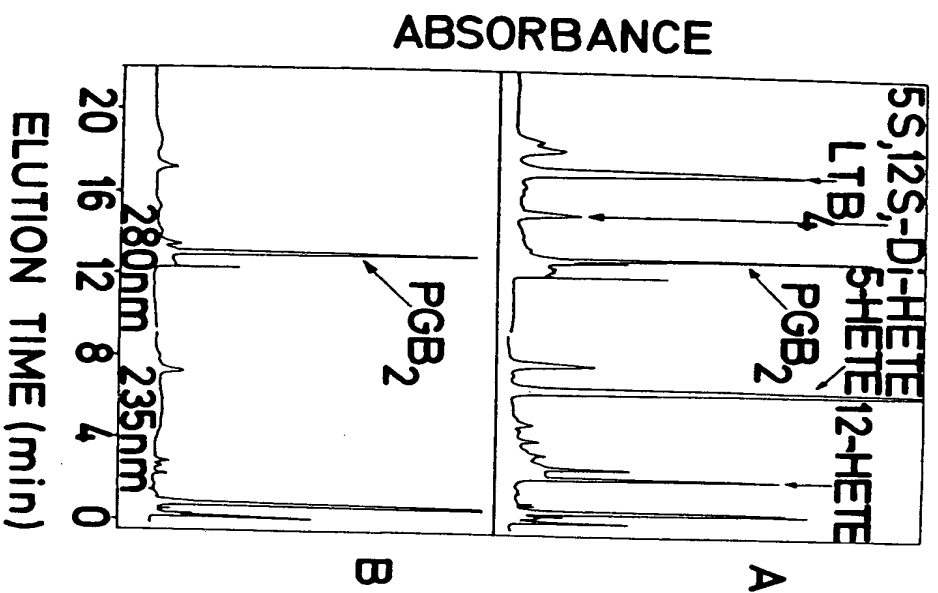


Fig. 2 :

Inhibition of porcine 5-lipoxygenase by 6,7,4'-trihydroxyisoflavan ; silica gel HPLC chromatograms of the products formed during a 5-min incubation of porcine periphera blood leukocytes (20 ml, 20 x 10⁶ cells/ml) with arachidonic acid, 100 μM, ionophore A23187, 6 μM, and ETYA, 10 μM (A) without inhibitor ; (B) with 6,7,4'-trihydroxyisoflavan 15 μM. Signal attenuation was 4 times higher at 235 than at 280 nm.

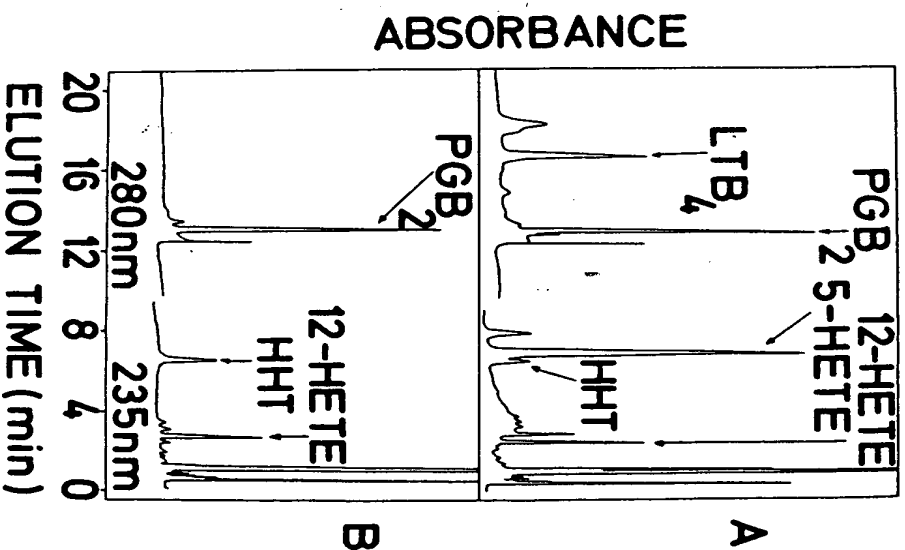


Fig. 3 :

Inhibition of human 5-lipoxygenase by 6,7,4'-trihydroxyisoflavan ; silica gel HPLC chromatograms of the products formed during a 5-min incubation of human periphera blood leukocytes (40 ml, 20 x 10⁶ cells/ml) with arachidonic acid, 100 μM, and ionophore A23187, 3 μM. (A) without inhibitor ; (B) with 6,7,4'-trihydroxyisoflavan , 15 μM. Signal attenuation was 4 times higher at 235 than at 280 nm.

inhibits the formation of 5-HETE and LTB_4 via the 5-lipoxygenase completely without inhibiting the production of HHT via the cyclooxygenase and with inhibiting the production of 12-HETE via the 12-lipoxygenase in the range of 50 % . The dose dependent inhibitory effect of 6,7,4'-trihydroxyisoflavan on human and porcine 5-lipoxygenase is identical (Fig. 6) .

Effect of 6,7,4'-trihydroxyisoflavan upon 12-lipoxygenase derived from human platelets

When arachidonic acid was incubated with human platelets, it was converted into three major compounds, 12-HETE, HHT and thromboxane B_2 (TXB_2) . Fig. 4 shows a typical radiochromatogram of arachidonic acid metabolites in the absence (control) and presence of two concentrations of 6,7,4'-trihydroxyisoflavan (100 μM and 800 μM) . At a concentration of 100 μM 6,7,4'-trihydroxyisoflavan inhibits the formation of 12-HETE completely without inhibiting the productions of TXB_2 and HHT at all.

Fig. 6 shows the dose dependent inhibitory effect of 6,7,4'-trihydroxyisoflavan upon the 12-lipoxygenase activity in the cytosol fraction of human platelets. When the IC_{50} values for 12-lipoxygenase in human platelets (22 μM) and 5-lipoxygenase in human and porcine leukocytes (1.6 μM) are compared a factor of 13.7 can be shown.

Effect of 6,7,4'-trihydroxyisoflavan on cyclooxygenase derived from ram vesicular gland microsomes and human platelets

With the specific incubation system for seminal vesicles microsomal cyclooxygenase the synthesis of HHT, PGD_2 , PGE_2

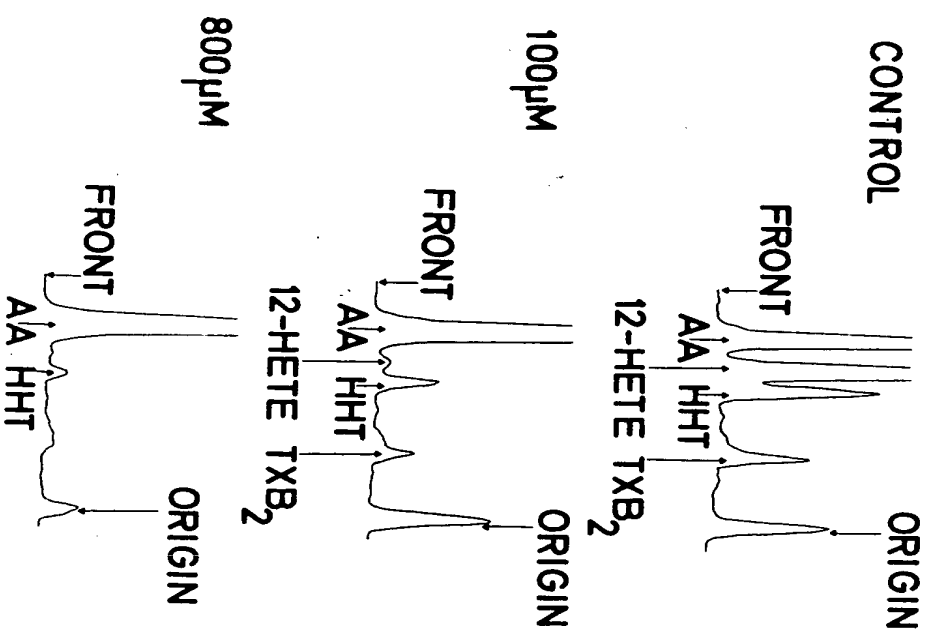


Fig. 4 :

Inhibition of human platelets 12-lipoxygenase and cyclooxygenase by 6,7,4'-trihydroxyisoflavan ; radiochromatogram scans of products obtained after incubation of human platelets (2 ml, 2×10^7 cells/ml) with (1- ^{14}C)-arachidonic acid without inhibitor (control), with 100 μM 6,7,4'-trihydroxyisoflavan , and with 800 μM 6,7,4'-trihydroxyisoflavan . Identity of the compounds was assessed by cochromatography of unlabeled reference markers. Plate was developed with solvent system A.

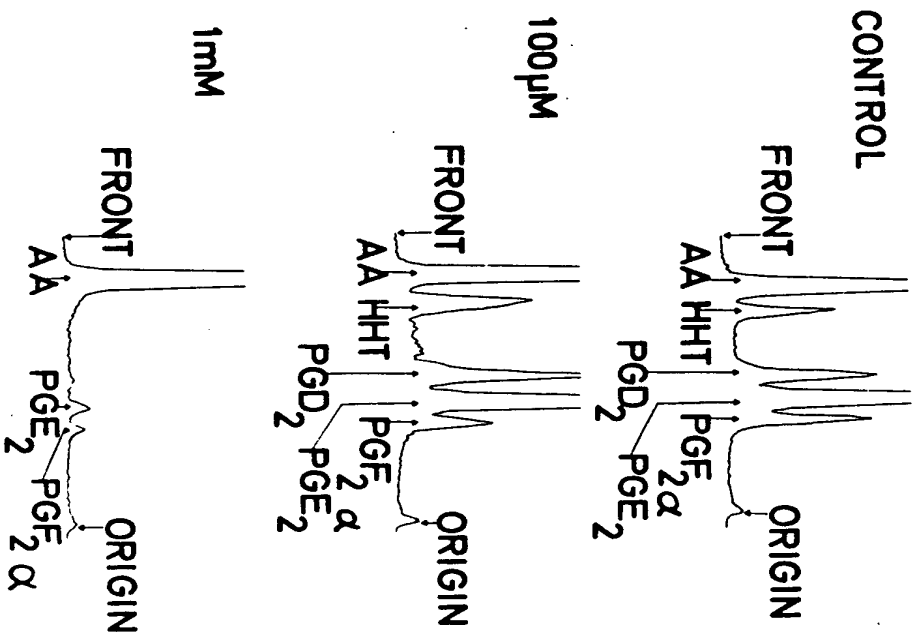


Fig. 5 :

Inhibition of ram cyclooxygenase by 6,7,4'-trihydroxyisoflavan ; radiochromatogram scans of products obtained after incubation of ram seminal vesicle microsomes with (1-¹⁴C)-arachidonic acid. Incubations were performed as described under "Methods". The concentration of 6,7,4'-trihydroxyisoflavan was 0 μM (Control), 100 μM, and 1 mM. Identity of the compounds was assessed by cochromatography of unlabelled reference markers. Plate was developed with solvent system B.

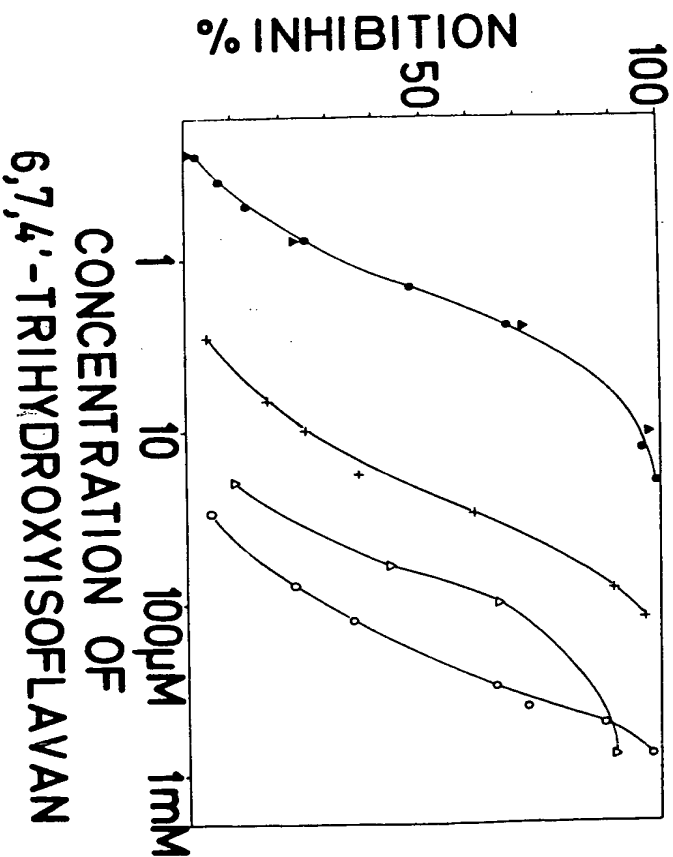


Fig. 6 :

Effect of 6,7,4'-trihydroxyisoflavan on various lipoygenases and cyclooxygenases. Reactions were carried out and quantitated as described under "Methods" (▲—▲) Human leukocyte 5-lipoxygenase; (●—●) porcine leukocyte 5-lipoxygenase; (+—+) human platelet 12-lipoxygenase; (○—○) human platelet cyclooxygenase; (○—○) cyclooxygenase from ram vesicular gland microsomes.

and PGF₂α has been demonstrated. Fig. 5 shows that 6,7,4'-trihydroxyisoflavan at a concentration of 1 mM almost completely inhibited cyclooxygenase from ram seminal vesicles. The dose dependent inhibitory effect of 6,7,4'-trihydroxyisoflavan on HHT and prostaglandin formation in this system is set out in Fig. 6. The IC₅₀ value for inhibition of cyclooxygenase from ram seminal vesicle microsomes is 125 and 9.1 times

higher than the IC_{50} values for inhibition of 5-lipoxygenase and human platelet 12-lipoxygenase respectively. A similar effect of 6,7,4'-trihydroxyisoflavan on the synthesis of TXB_2 and HHT in human platelets has been observed (Fig. 4, Fig. 6).

DISCUSSION

Several compounds have been reported to inhibit the 5-lipoxygenase reaction. BW-755C (16,17), nordihydroguaiaretic acid (18,19), benoxypofen (20) and 15-HETE (21,22) are non specific inhibitors of various lipoxygenases. ETVa strongly inhibits platelet 12-lipoxygenase and cyclooxygenase (23). There are a number of controversial data about the interaction of ETVa with leukotriene biosynthesis.

In porcine leukocytes ETVa is a potent inhibitor of 12-lipoxygenase but it activates 5-lipoxygenation (2). In human leukocytes 5-lipoxygenase is not inhibited directly by ETVa. ETVa suppresses the formation of 12-HPETE in platelets and therefore the activation of the leukocyte 5-lipoxygenase through this 12-lipoxygenase product (24). The lack of inhibitory effect of ETVa upon the 5-lipoxygenase has also been observed in rabbit peritoneal PMNL (25). In contrast, Sok et al. (26) found that ETVa and 5,6-dehydroarachidonic acid inhibit 5-lipoxygenase in RBL-1 cells, human leukocytes and guinea pig peritoneal PMNL. Bokoch and Reed (27) reported, that ETVa increases the 5-HETE production and inhibits LTB_4 formation in ionophore A23187 stimulated guinea pig peritoneal PMNL.

A recent report by Smith et al. (28) demonstrated that 6,9-deoxy-6,9-(phenylimino) - $\Delta^{6,8}$ -prostaglandin I_1 specifically inhibits LTB_4 synthesis in human neutrophils. The IC_{50} value of this inhibitor lies at about 2 μM . Yoshimoto et al. (29) showed 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone to inhibit 5-lipoxygenase in guinea pig peritoneal PMNL ($IC_{50} = 0.8 \mu M$). 12-Lipoxygenase and cyclooxygenases were not effected below 10 μM .

In our studies, we found that 6,7,4'-trihydroxyisoflavan is also a selective inhibitor for 5-lipoxygenase in human peritoneal blood leukocytes. At a concentration of 10 μM , formation of 5-lipoxygenase products was completely suppressed. In contrast only a 25 % inhibition of platelet 12-lipoxygenase and no inhibition of cyclooxygenase has been observed. The antioxidative and antihemolytic activities of 6,7,4'-trihydroxyisoflavan, isolated from fermented soybeans has been reported by Györfy et al. (30). Naim et al. (31) demonstrated an inhibitory effect of three isoflavones derived from soybeans (Glycitein, Genistein and Daidzein) upon soybean lipoxygenase at an inhibitor concentration of 1 mM. 6,7,4'-Trihydroxyisoflavan, the reduced form of 6,7,4'-trihydroxyisoflavan, showed no inhibition of soybean lipoxygenase and inhibition of auto-oxidation of arachidonic acid was observed at very high inhibitor concentrations only (>10 mM, unpublished data).

Among the flavonoids several flavones, flavonoles, flavanols and flavanonoles, especially Luteolin, 3', 4', 5', 7-tetrahydroxyethylrutin and Galangin (32) are inhibitors of soybean lipoxygenase and 12-lipoxygenase from rat lung and spleen. The IC_{50} values of these inhibitors are in the range of 30 - 200 μM . Most of them are also inhibitors of

ram seminal vesicle microsomes cyclooxygenase at comparable inhibitor concentrations (32,33).

There is no correlation between the radical-scavenging properties of these compounds and their inhibitory power upon cyclooxygenase activity (33).

For a number of years several flavonoids have been used as drugs affecting inflammatory responses in any way although their biochemical site of action and efficacy was rather unclear.

Our finding about the preferential inhibition of 5-lipoxygenase by 6,7,4'-trihydroxyisoflavan may explain these anti-inflammatory effects. 6,7,4'-trihydroxyisoflavan and other isoflavonoids, which are under further investigation, may be useful biochemical and pharmacological tools to elucidate the physiological and pathological functions of leukotrienes. Their clinical efficacy awaits further investigation.

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IDENTIFICATION OF LEUKOTRIENE D₄ SPECIFIC BINDING SITES IN THE MEMBRANE PREPARATION ISOLATED FROM GUINEA PIG LUNG

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Abstract

A radioligand binding assay has been established to study leukotriene specific binding sites in the guinea pig and rabbit tissues. Using high specific activity [³H]-leukotriene D₄ ([³H]-LTD₄), in the presence or absence of unlabeled LTD₄, the diastereoisomer of LTD₄ (5R,6S-LTD₄), leukotriene E₄ (LTE₄) and the end-organ antagonist, FPL 55712, we have identified specific binding sites for [³H]-LTD₄ in the crude membrane fraction isolated from guinea pig lung. The time required for [³H]-LTD₄ binding to reach equilibrium was approximately 20 to 25 min at 37°C in the presence of 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The binding of [³H]-LTD₄ to the specific sites was saturable, reversible and stereospecific. The maximal number of binding sites (B_{max}), derived from Scatchard analysis, was approximately 320±200 fmol per mg of crude membrane protein. The dissociation constants, derived from kinetic and saturation analyses, were 9.7 nM and 5.4 nM, respectively. The specific binding sites could not be detected in the crude membrane fraction prepared from guinea pig ileum, brain and liver, or rabbit lung, trachea, ileum and uterus. In radioligand competition experiments, LTD₄, FPL 55712 and 5R,6S-LTD₄ competed with [³H]-LTD₄. The metabolic inhibitors of arachidonic acid and SKF 88046, an antagonist of the indirectly-mediated actions of LTD₄, did not significantly compete with [³H]-LTD₄ at the specific binding sites. These correlations indicated that these specific binding sites may be the putative leukotriene receptors in the guinea-pig lung.

The abbreviations used are: 1, LTC₄, diastereoisomeric leukotriene C₄, 5R-hydroxy-6S and 5S-hydroxy-6R-S-glutathionyl-7E,9E,11Z, 14Z-cis-eicosatetraenoic acid; 2, LTD₄, or 5S,6R-LTD₄ the natural form LTD₄, 5S-hydroxy-6R-S-L-cysteinylglycyl-7E, 9E,11Z,14Z-cis-eicosatetraenoic acid; 3, 5R,6S-LTD₄, the unnatural form LTD₄, 5R-hydroxy-6S-S-L-cysteinylglycyl-7E, 11Z,14Z-cis-eicosatetraenoic acid; 4, LTE₄, diastereoisomeric LTE₄, 5R-hydroxy-6S and 5S-hydroxy-6R-S-L-cysteinyl-7E,9E,11Z, 14Z-cis-eicosatetraenoic acid; 5, SKF 88046, N,N'-bis [-(3-chloro-phenylamino)sulfonyl]-1,2,3,4-tetrahydroisoquinolinyl]-disulfonylimide; 6, HPLC, high pressure liquid chromatography; 7, Phenidone, 1-phenyl-3-pyrazolidone; 8, NDGA, nor-dihydroguaiaretic acid; 2,3-bis (3,4-dihydroxy-benzyl)-butane.

ANTIFUNGAL ACTIVITY OF SOYBEAN AND CHICKPEA ISOFLAVONES AND THEIR REDUCED DERIVATIVES

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(Revised received 5 March 1984)

Key Word Index—*Aspergillus ochraceus*; *Penicillium digitatum*; *Fusarium culmorum*; isoflavonoids; antifungal activity.

Abstract—The fungicidal activity of the isoflavones from soybean (*Glycine max*) and chickpea (*Cicer arietinum*) has been studied on three food and forage contaminating fungi, *Aspergillus ochraceus*, *Penicillium digitatum* and *Fusarium culmorum*. The reduced derivatives of the corresponding isoflavones—the isoflavanones and the isoflavans—were also included in the investigation. For the first time in a comparative study it is shown that isoflavones and isoflavanones are variable in their activity whereas the isoflavans are moderately active inhibitors of fungal growth.

INTRODUCTION

Isoflavonoids are naturally occurring substances possessing several biological properties [1]. In view of phytoalexin properties their fungicidal activity has attracted the attention of some research groups [2, 3]. Natural products, as potent fungal growth inhibitors, could be very useful substances in controlling plant diseases. In the present investigation, some isoflavones (1-4) from food sources have been tested for their fungicidal activity. Since most of the isoflavonoid phytoalexins possess a reduced isoflavone structure (Fig. 1), we have also included the corresponding isoflavanones (5-8) and isoflavans (9-12) of the above mentioned isoflavones in our investigation. The fungi of choice were *Aspergillus ochraceus*, *Penicillium digitatum* and *Fusarium culmorum* due to their food contaminating effects.

RESULTS AND DISCUSSION

As expected the fungi show a differential behaviour in regard to the applied isoflavonoids (Table 1 and Fig. 2).

While the growth of *A. ochraceus* is inhibited significantly by some isoflavonoids, *P. digitatum* and *F. culmorum* are inhibited as well as stimulated in their growth depending on the substances and their applied concentrations. The data are tabulated below; statistically significant percentage differences in weight as compared with the control are marked with an asterisk. The other values have been incorporated in the Table to indicate the trend of antifungal activity in the case of individual substances.

Among the isoflavonoids, the isoflavone 1 stimulates the growth of *P. digitatum* and *F. culmorum* at higher concentrations and shows very low inhibitory effect on *A. ochraceus* at all concentrations. The isoflavanone 5 is antifungal only to *F. culmorum*. The isoflavan 9 actively inhibits the growth of *A. ochraceus* and *F. culmorum* in more than one concentration. An inhibitory effect is also observed with *P. digitatum*.

Daidzein (2) inhibits *F. culmorum* in all applied concentrations. The isoflavanone 6 stimulates *F. culmorum*, but *P. digitatum* is inhibited significantly by the lowest concentration. The isoflavan 10 inhibits *P. digitatum* and *F. culmorum* only at higher concentrations.

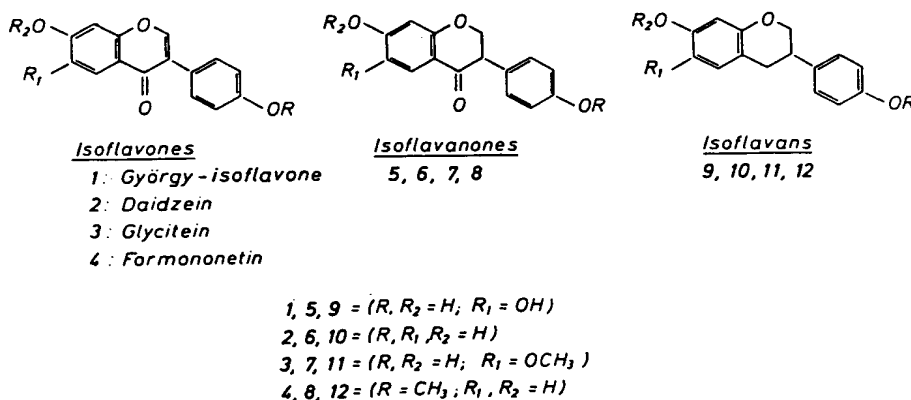


Fig. 1. Chemical structures of isoflavones and their reduced derivatives.

Table 1. Effect of isoflavonoids on mycelial growth of *A. ochraceus*, *P. digitatum* and *F. culmorum* [% difference in wt as compared with the control; (+) indicates growth stimulation and (-) growth inhibition]

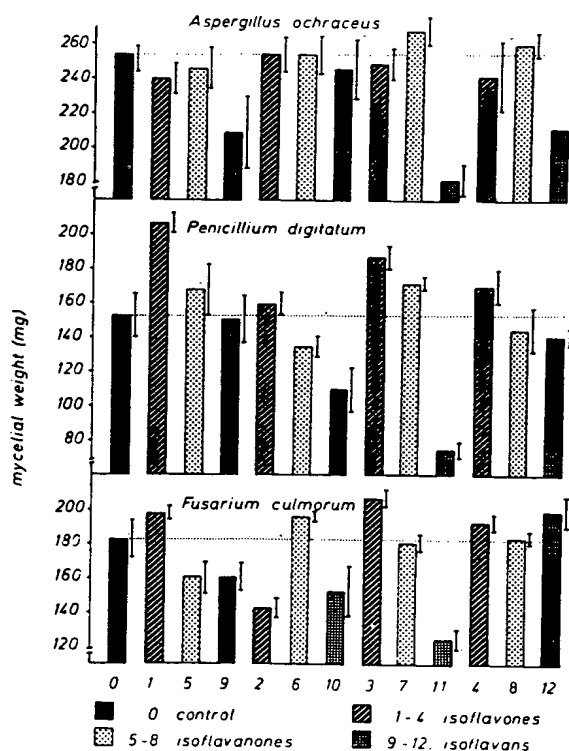
Isoflavonoids Concentration ($\times 10^{-4}$ mol/l.)	<i>A. ochraceus</i>			Fungi <i>P. digitatum</i>			<i>F. culmorum</i>		
	0.5	2.0	8.0	0.5	2.0	8.0	0.5	2.0	8.0
1	-5.0	-2.2	-5.5	+11.9	+31.1*	+35.4*	+1.6	+7.2*	+8.1*
5	+0.5	± 0	-3.2	-2.9	+2.1	+9.8	-7.4	-9.0	-11.9*
9	-19.7*	-15.4*	-17.7*	-8.9	-5.9	-0.9	-3.2	-13.0*	-11.9*
2	+1.2	+7.5	± 0	-6.5	-4.2	+4.3	-15.6*	-20.3*	-21.9*
6	+3.0	-3.2	-0.1	-13.5*	-10.9	-11.8	-1.0	+4.8	+7.0
10	+0.7	-9.3	-3.3	+3.2	+6.9	-28.2*	-5.1	+2.4	-16.5*
3	-8.8*	-4.0	-2.1	-7.4	+18.2*	+22.2*	+7.0	+19.2*	+12.9*
7	-5.4	+5.4	+5.5	-6.9	+18.0*	+12.5	-8.4	-2.6	-1.2
11	-3.3	-3.7	-28.2*	-27.4*	-36.7*	-50.8*	-4.0	+8.9	-31.9*
4	-0.3	-8.2	-4.5	+0.8	+6.8	+11.2*	+10.0	+5.8	+5.5
8	+1.2	+1.1	+2.4	+0.4	-4.3	-5.1	+4.3	+7.8*	+0.8
12	+7.0	-14.7*	-16.5*	+5.7	+2.5	-8.1	+4.8	+5.9	+8.7

Compounds 1-4, isoflavones; 5-8, isoflavanones; 9-12, isoflavans.

Glycitein (3) shows a fungal growth stimulation on *P. digitatum* and *F. culmorum*. The isoflavanone 7 also stimulates the growth of *P. digitatum*. In contrast, the isoflavan 11 is the best fungicide among all the 12 substances tested here. On malt extract agar plates it even produces a better inhibiting effect on *P. digitatum* and *F.*

culmorum (58.3 and 51.6%, respectively). In view of appreciable inhibitory activity, as found in liquid culture tests, this experiment was not performed with other substances.

The results with Formononetin (4) are contrary to the observations of Johnson *et al.* [4] and Van Etten [3]

Fig. 2. Mycelial dry wt (mg) of *A. ochraceus*, *P. digitatum* and *F. culmorum* on malt extract in presence of isoflavonoids at a concentration of 8×10^{-4} mol/l.

n [% difference in inhibition]

ilmorum

2.0	8.0
+7.2*	+8.1*
-9.0	-11.9*
13.0*	-11.9*
20.3*	-21.9*
+4.8	+7.0
+2.4	-16.5*
19.2*	+12.9*
-2.6	-1.2
-8.9	-31.9*
-5.8	+5.5
-7.8*	+0.8
-5.9	+8.7

actively). In view of no found in liquid culture performed with other

(4) are contrary to the [4] and Van Etten [3].

While they register a mild inhibitory activity with some fungi, in our case either an insignificant inhibition in *A. ochraceus* or a low growth stimulation is encountered in *P. digitatum* and *F. culmorum*. This discrepancy can be attributed to the variation in fungal species as well as to the differences in culture media. Among the reduced derivatives 8 and 12, the isoflavanone 8 shows virtually no inhibitory activity but the isoflavan 12 inhibits the growth of *A. ochraceus*.

The results of our investigation allow the conclusion that isoflavones and isoflavanones do not possess any remarkable fungicidal activity, whereas the isoflavans are comparatively good inhibitors of mycelial growth. This partially corroborates the hypothesis of Perrin and Cruickshank [5] that the isoflavans, due to a planar conformation, fit into the probable receptor sites in the cells of sensitive fungi [3]. The fungicidal activity of Daidzein (2) for *F. culmorum* or of the isoflavanones 5 and 6 for *P. digitatum* and *F. culmorum* respectively shows a deviation indicating that other factors may be important for the antifungal property of the isoflavonoids. Regarding the substitution pattern within the tested isoflavans, we observe that the methoxy group at the 6-position in 11 has a positive effect on fungal inhibition. In the case of the methoxy group at the 4'-position in 12 no inhibition is observed with *P. digitatum* and *F. culmorum*. The compound 10 without a methoxy group at the 4'-position shows a rather better activity against these fungi. It seems that the fungicidal property of the isoflavonoids is specific to individual fungi, substances and their concentrations and no absolute generalization is possible in this context. Ward *et al.* [6] came to the same conclusion in their investigation with differently substituted sesquiterpenes.

EXPERIMENTAL

Isoflavonoids. Compounds from a food source like soybean and chickpea were selected for the present study. 7,4'-Dihydroxy- and 7,4'-dihydroxy-6-methoxyisoflavones (2 and 3, respectively) occur in *G. max* [7]. György *et al.* [8] isolated 6,7,4'-trihydroxyisoflavone (1) from soybean fermented with *Rhizopus oligosporus*. 7-Hydroxy-4'-methoxyisoflavone (4) is found in *C. arletinum* [9]. As the quantities obtained from natural sources were meagre, the isoflavones were synthesized in our laboratory. In order to study the influence of gradual reduction on the antifungal activity, the isoflavones were catalytically (Pd-C; H₂) reduced to isoflavanones and isoflavans. The purity and authenticity of these products were verified by physical methods (NMR and MS).

Fungi and culture. The soil borne fungus *A. ochraceus* was isolated in Syria from fruits of Lady Fingers (*Hibiscus esculentus*) and kept in the fungus collection of the Institut für Pflanzenkrankheiten at Bonn. *P. digitatum* has been recently

isolated from lemon peel (*Citrus limon*) bought at the local market in Bonn. *F. culmorum* was found in panicles of winter barley (*Hordeum vulgare*) grown in Germany. Single spore cultures were prepared and preserved in a refrigerator at 8°. The effect of isoflavonoids on the mycelial growth of these fungi was investigated in liquid culture. The medium contained 30 g malt extract and 3 g peptone per l. distilled H₂O. Me₂CO served as solvent for the isoflavonoids. The solvent concn in the medium was maintained at a 1.1 % level. Medium (20 ml) including the particular isoflavonoid in proper concn was transferred to 100 ml Erlenmeyer flasks and inoculated with one small piece of mycelium (4 mm in diameter). The flasks were incubated at 23–25° on a reciprocal shaker for 7 days. Malt extract agar plates (30 g malt extract, 3 g peptone and 15 g agar per l. distilled H₂O) was also used to test the glycitein-isoflavan (11) with *P. digitatum* and *F. culmorum*. In this expt the isoflavan soln in a concn of 5×10^{-4} mol/l. was added to the medium after the sterilization process at a temperature of <60°. After inoculation the radial mycelial growth was measured for 7 days.

Concentrations of isoflavonoids. From phytoalexin experiments [3, 10] it is known that concns in the range of 10^{-5} to 10^{-3} mol/l. are effective in showing fungal inhibition. With 5,7-dihydroxyisoflavone as a reference substance the concns of 0.5, 2.0 and 8.0×10^{-4} mol/l. were determined to be suitable for our expts.

Evaluation of results. Round filter papers (Schleicher & Schüll No. 595) were weighed after drying for 24 hr at 105°. After filtration of the culture the residue was dried and weighed as described above. The difference of the weighings gave the dry wt of the fungi. The mean value of eight repetitions for each concn and fungus was used for calculation. The data were evaluated by analysis of variance. Probability of single differences was calculated at the 5% level.

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Genistein, a Specific Inhibitor of Tyrosine-specific Protein Kinases*

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Tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor, pp60^{src} and pp110^{src} was inhibited *in vitro* by an isoflavone genistein. The inhibition was competitive with respect to ATP and noncompetitive to a phosphate acceptor, histone H2B. By contrast, genistein scarcely inhibited the enzyme activities of serine- and threonine-specific protein kinases such as cAMP-dependent protein kinase, phosphorylase kinase, and the Ca²⁺/phospholipid-dependent enzyme protein kinase C. When the effect of genistein on the phosphorylation of the EGF receptor was examined in cultured A431 cells, EGF-stimulated serine, threonine, and tyrosine phosphorylation was decreased. Phosphoamino acid analysis of total cell proteins revealed that genistein inhibited the EGF-stimulated increase in phosphotyrosine level in A431 cells.

Tyrosine-specific protein kinase activity is known to be associated with oncogene products of the retroviral *src* gene family (1-3). This kinase activity is strongly correlated with the ability of retroviruses to transform cells, since mutants with reduced kinase activity have lower transforming efficiency, and mutants which lack tyrosine kinase activity are transformation-defective (4). Similar kinase activity is also associated with the cellular receptors for several growth factors such as EGF¹ (5), platelet-derived growth factor (6, 7), insulin (8, 9), and insulin-like growth factor I (10, 11). Therefore, it is possible that tyrosine phosphorylation plays an important role for cell proliferation and cell transformation.

According to this hypothesis, a specific inhibitor for tyrosine kinases could be an antitumor agent as well as a tool for

understanding the physiological role of tyrosine phosphorylation. Although not so specific for tyrosine kinases, several compounds have been reported to inhibit tyrosine kinase activity. A protease inhibitor *N*²-tosyl-L-lysyl chloromethyl ketone was demonstrated to inhibit tyrosine kinase activity associated with pp60^{src} and revert the effects of avian sarcoma virus transformation on cell morphology, adhesion, and glucose transport (12). A flavone quercetin was reported to inhibit the tyrosine kinase activity of pp60^{src} (13, 14) as well as the activities of cAMP-independent protein kinase (15), the Ca²⁺/phospholipid-dependent enzyme protein kinase C (16), phosphorylase kinase (17), Na⁺,K⁺-ATPase (18), and Ca²⁺,Mg²⁺-ATPase (19). More recently, amiloride, which is well known as an inhibitor for Na⁺,K⁺ antiporter (20-22), was shown to directly inhibit growth factor receptor tyrosine kinase activity (23).

In the search for specific inhibitors for tyrosine kinases, we have recently isolated an isoflavone compound genistein from fermentation broth of *Pseudomonas* sp. (24). In this study, we show that genistein is a highly specific inhibitor for tyrosine kinases but scarcely inhibits the activity of serine and threonine kinases and other ATP analogue-related enzymes *in vitro*. Furthermore, genistein was revealed to inhibit EGF-stimulated phosphorylation in cultured A431 cells.

EXPERIMENTAL PROCEDURES AND RESULTS²

DISCUSSION

In this study, we demonstrated that genistein inhibits the activities of tyrosine-specific protein kinases. Kinetic analysis revealed that inhibition of the EGF receptor kinase activity was competitive with ATP and that genistein leads to the formation of nonproductive enzyme-substrate complexes. Therefore, since Erneux *et al.* (39) have proposed that the reaction mechanism of the EGF receptor kinase is a sequential Ordered Bi Bi reaction with a peptide as the first substrate and ATP as the second, genistein could be expected to act uncompetitively with respect to a phosphate acceptor, histone H2B, *i.e.* genistein could bind to the enzyme only after histone combined (40). However, our results indicated that genistein was a noncompetitive inhibitor with respect to histone H2B. Since genistein bears no structural relationship to ATP, inhibition of the EGF receptor kinase activity by genistein may

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¹ The abbreviations used are: EGF, epidermal growth factor; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid (Miniprint); BSA, bovine serum albumin; RSV, Rous sarcoma virus; GA-FeSV, Gardner-Arnstein feline sarcoma virus; RIPA, radioimmune precipitation assay; NP-40, Nonidet P-40 (Miniprint).

² Portions of this paper (including "Experimental Procedures," "Results," Figs. 1-4, and Tables I-V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0280, cite the authors, and include a check or money order for \$7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

not be due to true competition for exactly the same site as that utilized by ATP. Thus, it would be possible that genistein binds in multiple places in the reaction pathway and, consequently, appears noncompetitive with respect to a phosphate acceptor. In this regard, it is intriguing that quercetin, which has a structure closely related to genistein, has also been reported to be competitive with ATP and noncompetitive with respect to histone (14), whereas amiloride, which resembles the structures of purines and pyrimidines, is competitive with ATP and uncompetitive with histone (23).

Genistein exhibited specific inhibitory activity against tyrosine kinases, that is, the EGF receptor kinase and pp60^{src} and pp110^{cas-les} kinases, but scarcely inhibited the activity of serine- and threonine-specific kinases such as cAMP-dependent protein kinase, protein kinase C, and phosphorylase kinase. These results are consistent with the fact that primary amino acid sequences of tyrosine kinases are closely related to each other but weakly homologous with the sequence of the catalytic subunit of cAMP-dependent protein kinase (1). Thus, genistein is not a mere ATP analogue, and can discriminate the differences in the catalytic site for ATP of these protein kinases. In addition, the activities of 5'-nucleotidase and phosphodiesterase were also poorly inhibited by genistein. The property of genistein to specifically inhibit tyrosine kinase activity is clearly different from that of a flavone quercetin, which has been reported to inhibit not only the tyrosine kinase activity associated with pp60^{src} (13, 14) but also the activities of protein kinase C (16), phosphorylase kinase (17), Na⁺,K⁺-ATPase (18), and 5'-nucleotidase (Table I, Mini-print). High specificity of genistein will be advantageous for utilizing this compound as a tool for elucidating the role of tyrosine phosphorylation in cells.

When the effect of genistein on the phosphorylation of the EGF receptor was examined in cultured A431 cells, EGF-stimulated increase of tyrosine phosphorylation was observed to decrease. EGF-induced increase in the level of cellular phosphotyrosine was also inhibited by the treatment of A431 cells with genistein. These results indicate that genistein inhibits the tyrosine kinase activity of the EGF receptor in intact A431 cells. Furthermore, genistein was found to inhibit EGF-stimulated serine and threonine phosphorylation of the EGF receptor. This may result from *in vivo* direct inhibition of serine and threonine kinase activity which is responsible for phosphorylation of the EGF receptor. Alternatively, inhibition of the EGF receptor-associated tyrosine kinase activity may block a putative cellular pathway regulating serine and threonine kinase activity that phosphorylates the EGF receptor. Precise mechanism of this effect is, however, not known at the present time and remains to be determined.

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Continued on next page.

All cells were metabolized for 6 h with [32 P]phosphate and then treated with and without NGF (100 ng/ml) for 15 min in the absence or presence of griseofulvin (40 μ g/ml). The NGF receptor was immunoprecipitated and analyzed as described in the legend to Fig. 6. The phosphorylated NGF receptor was eluted from the gel and subjected to scintillation counting. The results are shown in Table 1. The phosphorylation of the NGF receptor was increased 10-fold in the presence of this layer electrophoretic. Spots corresponding to phosphoserine, phosphothreonine, and phosphotyrosine were identified with ninhydrin and cut out from this layer plate. Radioactivity of each phosphoamino acid was counted and the results are presented in Table 1. The percentage of total radioactivity in the NGF receptor derived from untreated control cells, 100% represents 806 cpm.

TABLE IV
Phosphoamino acid analysis of the total cellular proteins of A431 cells
treated with genistein

	Total	Phospho- tyrosine	Phospho- threonine	Phospho- serine
Control	100	0.09	5.7	94
EGF	106	0.51	6.7	99
EGF + Genistein	94	0.16	5.5	88

A431 cells were labeled for 6 h with [32 P]phosphate and then treated with or without EGF (100 ng/ml) for 15 min in the absence or presence of genistein (40 μ M). The cell proteins extracted with 10% SDS and precipitated with 10% TCA were acid-hydrolyzed and subjected to two-dimensional separation by electrophoresis at pH 1.9 and pH 3.5. Spots corresponding to phosphoserine, phosphothreonine, and phosphotyrosine were identified with ninhydrin and cut out from the thin layer plates. Radioactivity of each phosphoamino acid was counted and the results are presented as percent of total phosphoamino acids in untreated control cells. 100% represents 756,000 cpm.

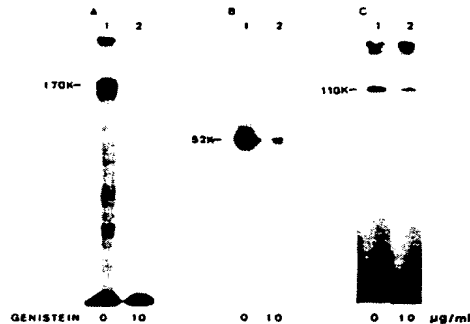


Fig. 1. Effect of genistein on the tyrosine kinase activity of the EGF receptor. (A) A431 cell membranes (A) were incubated with EGF (1 μ g/ml) and [γ - 32 P]ATP (40 μ M) in the absence (lane 1) or presence of 10 μ g/ml genistein (lane 2) for 5 min at 0°C. The reaction products were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography as described under "Experimental Procedures". (B) Tyrosine kinase activity of pp60^{src} immunoprecipitated from RSV-transformed Y1 cells (B) and pp60^{src} immunoprecipitated from Feline sarcoma virus-transformed Y1 cells (C) were carried out at 25°C for 5 min and analyzed similarly.

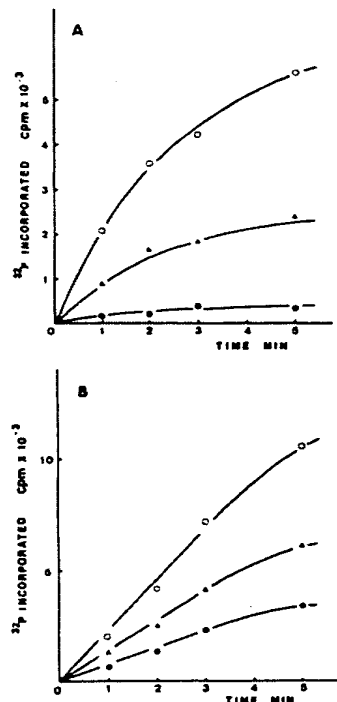


Fig. 2. Effect of genistein on the time course of autophosphorylation of the EGF receptor. (A) and (B) A431 cell membranes were incubated with EGF (1 μ g/ml), [γ - 32 P]ATP (40 μ M) and sodium vanadate (100 μ M) in the absence (○) or presence of genistein (A: 1 μ g/ml; B: 10 μ g/ml; C: 100 μ g/ml) for the indicated time periods at 0°C. In panel (B), histone H2B (500 μ g/ml) was included in the reaction mixture. The reaction products were analyzed by SDS polyacrylamide gel electrophoresis and the bands of the EGF receptor (A) or histone H2B (B) were excised from the gels and the radioactivity was counted with a liquid scintillation counter as described under "Experimental Procedures".

TABLE V
Effect of genistein on [32 P]-EGF binding to A431 cells.

Genistein μ g/ml	[32 P]-EGF bound cpm
0	37483
10	36410
40	37077
100	30475

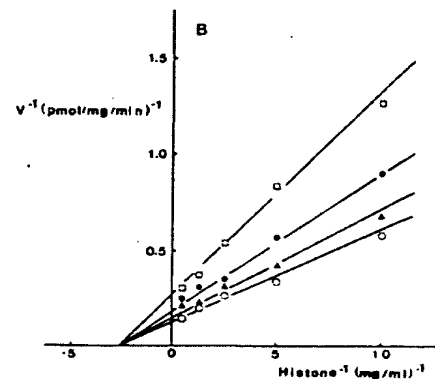
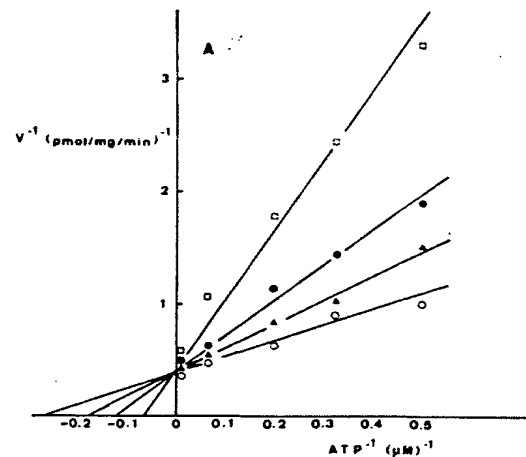


Fig. 3. Effect of genistein on the kinetics of the EGF receptor kinase activity. (A) A431 cell membranes were incubated with EGF (1 μ g/ml), sodium vanadate (100 μ M), histone H2B (200 μ g/ml) and various concentrations of [γ - 32 P]ATP in the absence (○) or presence of genistein (A: 1 μ g/ml; B: 10 μ g/ml; C: 100 μ g/ml) for 1 min at 0°C. The reaction products were analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography. The bands of histone H2B were excised from the gels and the radioactivity was counted with a liquid scintillation counter. (B) The EGF receptor kinase activity was assayed with various concentrations of histone H2B in the absence (○) or presence of genistein (A: 1 μ g/ml; B: 10 μ g/ml; C: 100 μ g/ml) for 1 min at 0°C. The concentration of [γ - 32 P]ATP was 40 μ M.

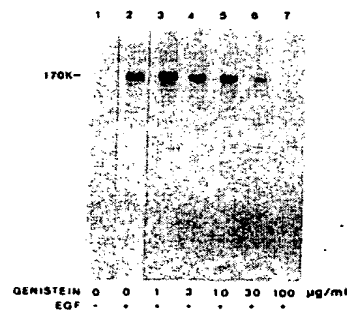


Fig. 4. Effect of genistein on the phosphorylation of the EGF receptor in vivo. A431 cells were labeled for 6 h with [32 P]phosphate and then EGF (100 ng/ml) and various concentrations of genistein were added for the last 15 min. The detergent lysates of the labeled cells were immunoprecipitated with anti-EGF receptor antibody and protein A-Sepharose. The immunoprecipitates were then analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography.

Oncogenes and Signal Transduction

Review

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Oncogenes were first described as retrovirus-encoded genes that produced tumors in birds and rodents. These genes were then shown to be dominant mutated forms of host genes (proto-oncogenes) that had been picked up by the retroviruses (Bishop, 1991 [this issue of *Cell*]). Most proto-oncogenes are thought to encode proteins that are involved in the cascade of events by which growth factors stimulate normal cell division.

Oncogenes described to date encode proteins that fall into four classes: growth factors (e.g., *v-sis*), growth factor receptors (*v-erbB*, *v-fms*, *v-kit*), transducers of growth factor responses (*v-src*, *v-ras*, *v-raf*), and transcription factors that mediate growth factor-induced gene expression (*v-jun*, *v-fos*). In general, the transcription factor-type oncogenes appear to act in cooperation with oncogenes from the other categories to accomplish transformation (Hunter, 1991 [this issue]).

Transformation is also facilitated by recessive mutation of certain genes, which have thus been termed anti-oncogenes or tumor suppressor genes (Marshall, 1991 [this issue]). Several DNA tumor viruses (SV40, papilloma, polyoma, adenovirus) produce proteins that associate with products of the retinoblastoma (*RB*) and *p53* tumor suppressor genes. Although the functions of these genes are not yet fully understood, their protein products clearly play critical roles in growth regulation since diverse viruses accomplish cell immortalization by targeting the same two proteins (Buchkovich et al., 1990; Weinberg, 1990). The recent discovery that the protein encoded by the neurofibromatosis gene (an implied anti-oncogene) is homologous to *ras* GTPase activating protein (*ras* GAP), a protein that down-regulates the activity of the *c-ras* proto-oncogene, suggests that anti-oncogenes may act by directly attenuating proto-oncogene functions (Xu et al., 1990).

In the past 2 years some critical connections have also been made between oncogenes and genes that regulate progression through the normal cell cycle (Barrett et al., 1990; Maller, 1990; Roy et al., 1990). Notably, *c-mos* was found to encode a component of the cytoskeletal factor that blocks a new round of cell division in *Xenopus* oocytes (Sagata et al., 1989). The serine kinase encoded by the *c-mos* proto-oncogene was shown to phosphorylate cyclin B in vitro (Roy et al., 1990). *p34^{cdc2}*, a serine/threonine kinase centrally involved in cell cycle progression, was also shown to be phosphorylated on tyrosine in a cell cycle-dependent manner, implicating the protein-tyrosine kinase family of proto-oncogenes in cell cycle regulation (Draetta

et al., 1988). In addition, *p34^{cdc2}* appears to phosphorylate *pp60^{c-src}* in a cell cycle-dependent manner (Morgan et al., 1989; Shenoy et al., 1989).

Thus, after years of slow progress in understanding the function of oncogenes, a plethora of biochemical connections between the proteins encoded by these genes have recently been made. This progress has allowed a better understanding of the biochemical mechanisms of cell transformation. In addition, the mechanism by which growth factors signal mitogenic responses to the nucleus of normal cells has become more clear. One major breakthrough in this area has been the realization that many growth factors trigger changes in the cytosolic domains of their receptors that result in the recruitment of critical signal-transducing enzymes from the cytosol to the inner surface of the plasma membrane. The growth factor-dependent association of these transducers with the receptors has allowed them to be purified and identified. Thus, some of the primary responses to growth factor stimulation have been determined. Not surprisingly, oncogenes activate some of the same transducing enzymes as growth factor receptors.

The purpose of this review is to incorporate recent discoveries into a general biochemical pathway by which the protein products of oncogenes send signals from the cell surface to the nucleus. The protein-tyrosine kinase oncogenes will be the primary focus of the review. However, biochemical connections between the protein-tyrosine kinases and oncoproteins of the Ras, Raf, Fos, Jun, and Rel families as well as the protein kinase C family are also discussed. For more comprehensive discussions of connections between oncogenes and cell cycle genes see Maller (1990) and Lewin (1990).

Nonreceptor Protein-Tyrosine Kinases

A large family of protein-tyrosine kinases has been revealed over the past 10 years (reviewed by Hanks et al., 1988). Many of these enzymes were discovered as products of retrovirus-encoded oncogenes. These kinases fall into two families: the transmembrane receptor family and the cytosolic nonreceptor family. *pp60^{c-src}* is the prototype of the latter family and the best characterized of the protein-tyrosine kinases.

Structure of *pp60^{c-src}*

All the protein-tyrosine kinases have sequence homology over a stretch of approximately 300 amino acids (sometimes interrupted; see below) that has been defined as the kinase domain (Hanks et al., 1988). The Src subfamily has additional regions of homology not found in the receptor family (Figure 1). These regions include a short amino-terminal sequence required for addition of myristic acid and two additional domains named Src homology 2 and 3 (SH-2 and SH-3). The myristic acid addition is required for membrane localization (Cross et al., 1985; Buss and Sefton, 1985; Kamps et al., 1985). The association with

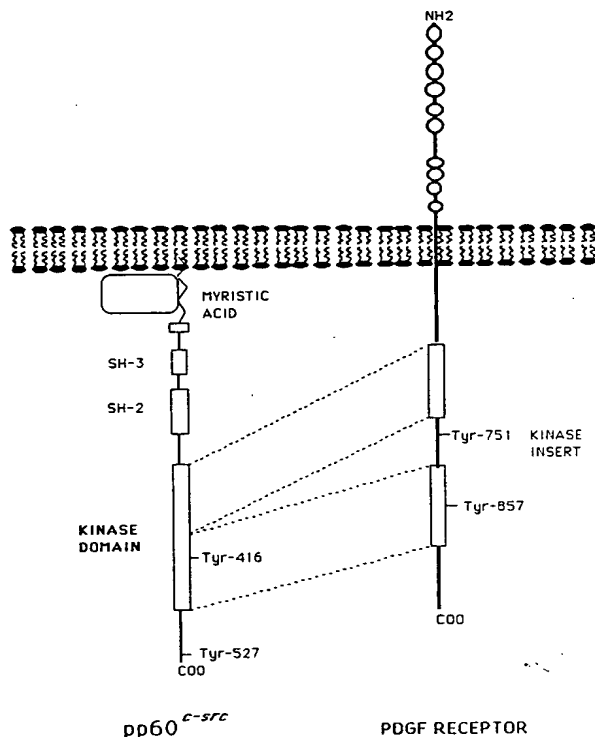


Figure 1. Examples of the Receptor and Nonreceptor Families of Protein-Tyrosine Kinases

All protein-tyrosine kinases have sequence similarity over the kinase domain (Hanks et al., 1988). The Src-like subfamily has additional regions of homology labeled SH-2 and SH-3 that are not conserved in the receptor family (see text). Proteins of the Src family have a consensus sequence at the amino terminus that results in attachment of myristic acid. A recently discovered 32 kd protein binds myristoylated pp60^{c-src} and causes localization at the inner surface of the plasma membrane (Resh and Ling, 1990). Proteins of the receptor family have an amino-terminal extracellular domain that binds ligand and a single stretch of approximately 20 hydrophobic amino acids that span the membrane bilayer.

the membrane is not merely a consequence of the hydrophobicity of the myristic acid: a membrane protein that provides a receptor for myristoylated peptides was recently described (Resh and Ling, 1990). Association with the plasma membrane via myristic acid addition to the amino terminus is also common to diverse families of proteins unrelated to tyrosine kinases.

The SH-2 and SH-3 domains have also been discovered in proteins otherwise unrelated to protein-tyrosine kinases. Included are phospholipase C- γ (PLC- γ) (Stahl et al., 1988; Suh et al., 1988a, 1988b), *ras* GAP (McCormick, 1989), and the *crk* oncogene product (Mayer et al., 1988). All the proteins in this interesting group associate with activated protein-tyrosine kinases in intact cells. Most intriguing is the retrovirus-encoded *gag-crk* oncogene. This gene encodes a small protein that consists of very little sequence besides a viral *Gag* sequence and an SH-2 and SH-3 domain. Regions of similarity to the SH-3 domain (but not SH-2 domain) have been detected in actin-binding

proteins including myosin, spectrin, and a yeast cytoskeletal protein (Drubin et al., 1990), raising the possibility that this domain is important for association with the cytoskeleton. Interestingly, the c-Abl protein is associated with actin filaments, and deletion of the SH-3 domain eliminates this association and causes transformation (Jackson and Baltimore, 1989; Van Etten et al., 1989).

Mechanisms for Regulation of the Nonreceptor Family of Protein-Tyrosine Kinases

The normal cellular products of the *src* family of proto-oncogenes have relatively low levels of protein-tyrosine kinase activity. Although these proteins have no extracellular domain for direct binding to growth factors, there is evidence that these kinases are activated by growth factors and other cellular activators. In platelets, thrombin activates protein-tyrosine kinases of the Src family (Ferrell and Martin, 1988, 1989; Golden and Brugge, 1989; Gutkind et al., 1990; Rendu et al., 1989). Platelet-derived growth factor (PDGF) activates the protein-tyrosine kinase activity of pp60^{c-src} in fibroblasts (Gould and Hunter, 1988; Kypta et al., 1990). Protein-tyrosine phosphorylation can be increased in some cells by GTP- γ -S (Nasmith et al., 1989) and by activators of the protein-serine/threonine kinase, protein kinase C (PKC) (Kazlauskas and Cooper, 1988). Thus, it is possible that G proteins and/or PKC is an intermediate in the signal cascade from receptor to protein-tyrosine kinase activation. However, in some cases enzymes of the Src family directly associate with the cytosolic domain of transmembrane receptors (see CD4/CD8 association with pp56^{c-*lck*} below).

Carboxy-Terminal Phosphorylation

Comparison of transforming and nontransforming mutants of pp60^{c-src}, pp60^{v-src}, and other members of this family has provided clues to the biochemical mechanism by which protein-tyrosine kinase activity is regulated. The first important clue was the discovery that pp60^{c-src} is phosphorylated in vivo at a tyrosine residue near the carboxyl terminus (Tyr-527) in a region outside of the kinase domain (Cooper et al., 1986; Schuh and Brugge, 1988). pp60^{v-src} lacks this tyrosine residue, and mutation of this tyrosine to a phenylalanine increases the protein-tyrosine kinase activity and the transforming activity of pp60^{c-src} (Cartwright et al., 1987; Kmiecik and Shalloway, 1987; Piwnicka-Worms et al., 1987; Reynolds et al., 1987).

Other proto-oncogenes in this family have potential tyrosine phosphorylation sites at analogous locations, and these regions are typically deleted in the retrovirus-captured genes. Phosphorylation of pp60^{c-src} at Tyr-527 is apparently mediated by a distinct protein-tyrosine kinase rather than by autophosphorylation (Okada and Nakagawa, 1989). Thus, one potential way to activate pp60^{c-src} and other members of this family is to dephosphorylate at the carboxy-terminal phosphotyrosine. This possibility is being explored, especially as a mechanism of action of the lymphocyte CD45 "receptor-like" protein-phosphotyrosine phosphatase (Mustelin and Altman, 1990; Ostergaard and Trowbridge, 1990).

The importance of phosphotyrosine 527 in regulating pp60^{c-src} protein-tyrosine kinase activity is further sup-

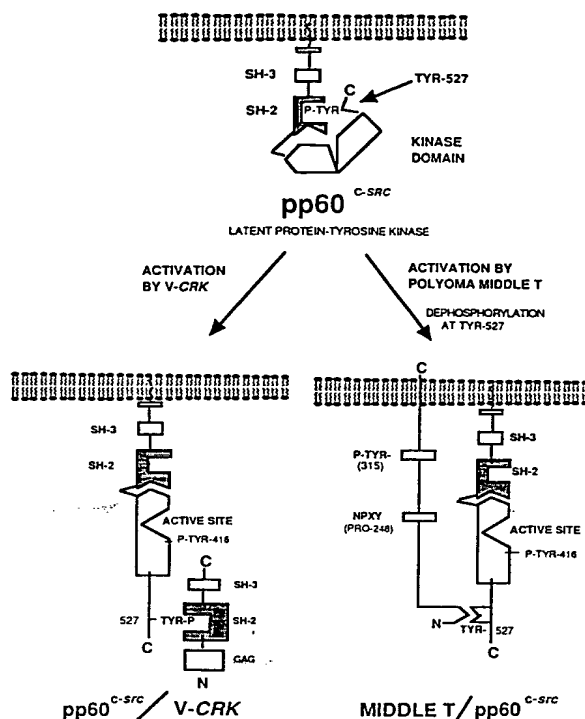


Figure 2. Model for Regulation of the Protein-Tyrosine Kinase Activity of pp60^{c-src} by Carboxy-Terminal Phosphorylation on Tyrosine

In normal cells, pp60^{c-src} is phosphorylated at tyrosine-527 and has very low protein-tyrosine kinase activity. The diagram at top indicates that phosphotyrosine-527 associates with the SH-2 domain of the same pp60^{c-src} molecule to form a structure with low protein-tyrosine kinase activity. This model is based on results from Hanafusa's laboratory indicating that SH-2 domains specifically interact with tyrosine-phosphorylated proteins (see text). Significantly, pp60^{v-src} lacks tyrosine-527.

The diagram at bottom right indicates that the polyomavirus transforming protein, middle t, associates with pp60^{c-src} in such a way as to prevent phosphorylation of tyrosine-527 and thereby blocks the head-to-tail association. This results in activation of pp60^{c-src} protein-tyrosine kinase activity. The association involves the amino terminus of middle t (Cook and Hassell, 1990) and the carboxyl terminus of pp60^{c-src} (Piwnica-Worms et al., 1990). Also indicated are the major site of tyrosine phosphorylation of middle t (Tyr-315), the major autophosphorylation site of pp60^{c-src} (Tyr-416), and an NPXY sequence in middle t (see text).

The diagram at bottom left suggests a possible mechanism for activation of protein-tyrosine kinases by the v-crk oncogene product. This protein has an SH-2 domain that might bind to phosphotyrosine-527 of pp60^{c-src} and thereby cause activation (see text).

ported by studies with the polyomavirus transforming gene, middle t (Figure 2). This gene encodes a 58 kd protein that forms a 1:1 complex with certain proteins in the Src family, especially pp60^{c-src}, pp59^{c-fyn}, and pp60^{c-yes} (Courtneidge and Smith, 1983; Cheng et al., 1988a; Kypta et al., 1988; Kornbluth et al., 1990). The protein-tyrosine kinase activity of pp60^{c-src} is activated by formation of this complex (Bolen et al., 1984; Courtneidge, 1985). The region of pp60^{c-src} implicated in binding of middle t is proximal to Tyr-527 (Cheng et al., 1988b, 1989; Piwnica-Worms et al., 1990), and this residue fails to be phosphorylated

in the complex (Cartwright et al., 1986; Cheng et al., 1989). Thus, middle t appears to activate pp60^{c-src} at least in part by preventing phosphorylation of Tyr-527.

The SH-2 Domain as a Phosphotyrosine-Binding Site. In attempts to understand the mechanism of cell transformation by gag-crk, it was discovered that this gene activates cellular protein-tyrosine kinase activity (Mayer et al., 1988) and that immunoprecipitates of p47^{gag-crk} protein from transformed cells contain a collection of tyrosine-phosphorylated proteins similar to those found in anti-phosphotyrosine antibody immunoprecipitates (Matsuda et al., 1990; Mayer and Hanafusa, 1990). p47^{gag-crk} also specifically associated with pp60^{v-src} but failed to associate with pp60^{c-src} if autophosphorylation at Tyr-416 of pp60^{v-src} was prevented (Matsuda et al., 1990). Mutational studies of Gag-Crk indicated that the only region of the avian protein essential for transformation or for association with the tyrosine-phosphorylated proteins was the SH-2 domain. These results indicate that the SH-2 domain of p47^{gag-crk} has specificity for tyrosine-phosphorylated regions of proteins. A labeled form of the SH-2 domain of the Abl protein binds to a similar set of SDS-denatured cellular polypeptides, as does an anti-phosphotyrosine antibody. Pretreatment of the cellular proteins with a phosphotyrosine phosphatase prevents the binding (Mayer et al., 1991). These results support the idea that the SH-2 domain binds to tyrosine-phosphorylated proteins and indicates that this association readily occurs with proteins that have been denatured in SDS.

A simple model has therefore been proposed to explain how phosphorylation of Tyr-527 inhibits the protein-tyrosine kinase activity of pp60^{c-src} (Matsuda et al., 1990) (see upper half of Figure 2). In this model, the SH-2 domain is a binding site for specific peptide sequences containing phosphotyrosine. This association is regulated by tyrosine phosphorylation and dephosphorylation. Specificity of association of a particular SH-2 domain with a particular phosphopeptide is provided by the amino acid residues surrounding the phosphotyrosine (and perhaps also by the SH-3 domain; see below). The model in Figure 2 proposes that when Tyr-527 of pp60^{c-src} is phosphorylated, this domain folds into the SH-2 domain of the same molecule in a head-to-tail self-association that inhibits protein-tyrosine kinase activity. There is evidence that the amino-terminal and carboxy-terminal regions of pp60^{v-src} are closely associated in the native state (McCarley et al., 1987). This model is analogous to autoinhibitory-domain models that have been proposed to explain regulation of protein-serine kinases (Soderling, 1990).

The abilities of p47^{gag-crk} and polyoma middle t to activate cellular protein-tyrosine kinases might also be explained by this model. For example, p47^{gag-crk} could activate pp60^{c-src} by competing with the intrinsic SH-2 domain of pp60^{c-src} for association with phosphotyrosine-527. This would free the SH-2 domain of pp60^{c-src}, allowing activation of protein-tyrosine kinase activity and association with critical targets (see bottom of Figure 2). Thus far there is no evidence that p47^{gag-crk} can associate with Tyr-527 in vivo. As discussed above, association of polyoma middle t with pp60^{c-src} reduces phosphoryla-

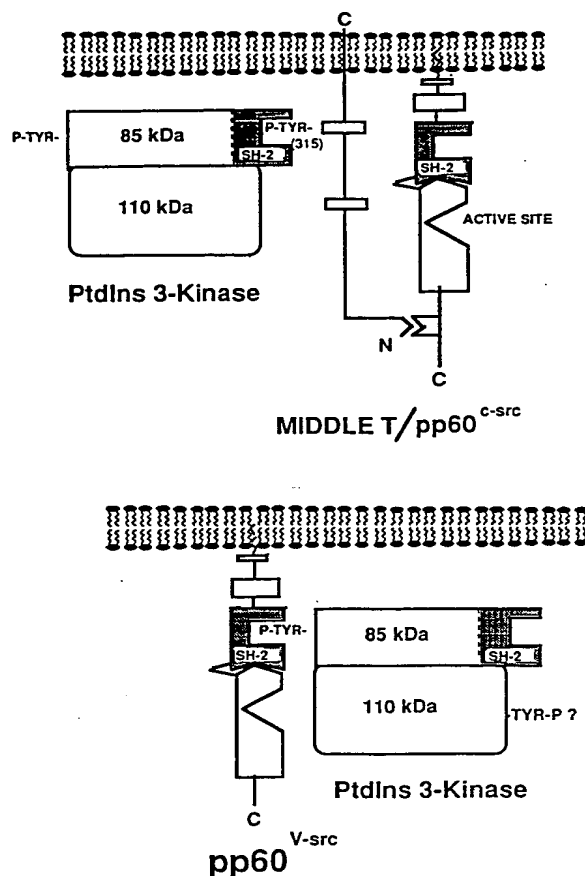


Figure 3. Involvement of SH-2 Domains in the Association of PtdIns 3-Kinase with Middle t-pp60^{c-src} and with pp60^{v-src}

The PtdIns 3-kinase is a heterodimer of 85 kd and 110 kd proteins (Carpenter et al., 1990). The 85 kd subunit has SH-2 domains and is phosphorylated on tyrosine in middle t-transformed cells (see text). The upper diagram suggests that phosphorylation of middle t at Tyr-315 creates a binding site for the SH-2 domain of the 85 kd subunit of PtdIns 3-kinase. The lower diagram suggests that association of PtdIns 3-kinase with pp60^{v-src} occurs in a reciprocal fashion: pp60^{v-src} first phosphorylates the PtdIns 3-kinase and then the phosphoprotein associates with the SH-2 domain of pp60^{v-src} (see text)

tion at Tyr-527, and this would also prevent the head-to-tail self-association (bottom of Figure 2).

The results of mutational studies of the SH-2 domain of pp60^{c-src}, pp60^{v-src}, and pp130^{v-ps} can be interpreted in terms of this model. Deletion of a large region of pp60^{c-src} including the SH-2 domain compromises the ability of pp60^{c-src} to transform when overproduced (Nemeth et al., 1989). This result could indicate that the SH-2 domain plays a positive role in substrate utilization by pp60^{v-src} or that the mutant protein fails to fold properly. Deletion of a phenylalanine from a conserved region of the SH-2 domain reduces the ability of pp60^{v-src} to phosphorylate substrates in intact cells and to transform mammalian but not chicken fibroblasts (Verderame et al., 1989). This result argues that the SH-2 domain is important for sub-

strate utilization by pp60^{v-src}. Consistent with this idea, other mutations in the SH-2 domain of activated pp60^{v-src} have been shown to affect association with specific cellular proteins and/or cell transformation (Hirai and Varmus, 1990; Reynolds et al., 1989; Wang and Parsons, 1989; Wendler and Boschelli, 1989). Similarly, mutations in the SH-2 domain of pp130^{v-ps} can cause either positive or negative effects on protein-tyrosine kinase activity and can also affect specific substrate recognition in vivo (Koch et al., 1989).

The surprising finding that a single amino acid substitution in a conserved region of the SH-2 domain of pp60^{c-src} is able to activate protein-tyrosine kinase activity, cause association with one of its targets (phosphatidylinositol 3-kinase; see below), and enhance cell transformation without an increase in phosphorylation of either Tyr-416 or a decrease in phosphorylation of Tyr-527 (O'Brien et al., 1990) could also be explained by the models in Figures 2 and 3. One could argue that the mutations decrease the affinity of the SH-2 domain for the phosphotyrosine-527 region, thereby freeing this domain to activate the protein-tyrosine kinase activity and to associate with critical targets.

These results not only suggest a mechanism for regulation of protein-tyrosine kinase activity but also provide new insight into the role of autophosphorylation of protein-tyrosine kinases in signal transduction. The prevalence of autophosphorylation (or transphosphorylation of homodimers) as the major activity observed in vivo has distinguished the protein-tyrosine kinases from the serine/threonine kinases. In fact, the difficulty in finding relevant protein-tyrosine kinase substrates other than the kinases themselves has been a hallmark of this field. The models in Figures 3 and 4 suggest that autophosphorylation alone could be important by providing binding sites for specific cytosolic proteins containing SH-2 domains. In this model, the protein-tyrosine kinase provides the critical gathering place for signal-transducing molecules. Strong support for this model is provided by a recent demonstration that isolated SH-2 domains can bind to activated receptor-type protein-tyrosine kinases (Anderson et al., 1990). This model is discussed in more detail below.

Receptors That Bind Directly to Src-like

Protein-Tyrosine Kinases

Studies of the CD4 and CD8 lymphocyte cell surface proteins have provided a simple model for activation of a protein-tyrosine kinase of the Src family by extracellular molecules. The Src-like tyrosine kinase p56^{lck} is associated with the cytosolic domain of both of these proteins. Stimulation of the appropriate T cells with antibodies directed against CD4 results in activation of p56^{lck} (Veillette et al., 1988). Similar results are found using antibodies against CD8 and T cells containing this antigen (Veillette et al., 1988; reviewed by Rudd et al., 1989). A short region of the carboxy-terminal cytosolic domain of these two proteins has been implicated in the association with p56^{lck} (Barber et al., 1989; Shaw et al., 1990; Turner et al., 1990). The association occurs via an amino-terminal region of p56^{lck} that is not conserved in other protein-tyrosine kinases (Shaw et al., 1989; Turner et al., 1990;

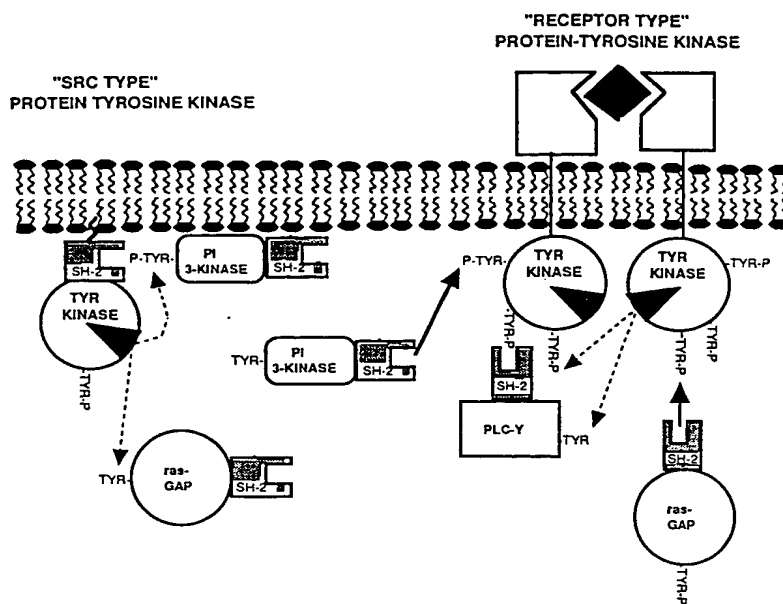


Figure 4. Recruitment of Cytosolic Enzymes with SH-2 Domains to Activated Protein-Tyrosine Kinases

This model is an extension of the model in Figure 3. Receptor-type protein-tyrosine kinases undergo ligand-induced dimerization, resulting in cross-phosphorylation of the subunits on tyrosine residues. The multiple tyrosine-phosphorylated regions of the receptors provide specific binding sites for cytosolic enzymes containing SH-2 domains. The sequence surrounding the phosphotyrosine provides recognition of specific SH-2 domains. Once associated, these proteins are substrates for the receptor protein-tyrosine kinase. Tyrosine phosphorylation may modulate the activities of these proteins (see text). Recruitment alone may also be important since the substrates for PtdIns 3-kinase and PtdIns-specific PLC- γ are membrane lipids and the target for GAP is p21^{ras}, which also resides at the plasma membrane (see text).

Vega et al., 1990). Extrapolating from the model in Figure 2, one could propose that upon binding of extracellular ligand, CD4 induces a conformational change at the amino terminus of p56^{lck} that weakens the association between the SH-2 domain and the carboxy-terminal phosphotyrosine, thereby opening the enzyme for substrate utilization. Dephosphorylation of the carboxy-terminal tyrosine would not be required in this model. Further studies are necessary to elucidate this pathway.

The T cell receptor also activates a Src-like tyrosine kinase upon stimulation with antibodies. This receptor directly binds to the c-Fyn tyrosine kinase (Samelson et al., 1990), and the ζ subunit of the associated CD3 complex becomes phosphorylated on tyrosine. The mechanism of activation of c-Fyn is not yet clear. There is also evidence for activation of p56^{lck} by antibodies against the T cell receptor and by antibodies against CD2 (Danielian et al., 1989).

Recently, evidence for regulation of Src-like protein-tyrosine kinases by direct association with the PDGF receptor has been found (Kypta et al., 1990). pp60^{c-src} associates with the PDGF receptor in a PDGF-dependent manner, and the protein-tyrosine kinase activity of pp60^{c-src} from PDGF-stimulated cells is increased. It is possible that autophosphorylation of the PDGF receptor at a tyrosine residue forms a binding site for the SH-2 domain of pp60^{c-src}, causing recruitment and activation (compare Figure 3). Alternatively, pp60^{c-src} may associate with the PDGF receptor via an intermediary protein such as phosphatidylinositol 3-kinase (PtdIns 3-kinase), forming a bridge between these two protein-tyrosine kinases. This possibility is discussed more below.

pp60^{c-src} and other members of this family are also phosphorylated on serine/threonine residues in vivo, and it is likely that these modifications have some effect on lo-

cation, association with targets, or regulation of kinase activity (Luo and Sefton, 1990; Patschinsky et al., 1986).

Primary Targets of Nonreceptor Protein-Tyrosine Kinases

Until recently, relevant cellular substrates of the protein-tyrosine kinase activity of the Src-like enzymes have been elusive. The low level of in vivo tyrosine phosphorylation, when compared with the rapid and pleiotropic cellular responses observed, argues that the critical cellular targets of protein-tyrosine kinases are likely to be amplifiers. Enzymes with potential regulatory activities have been found to bind tightly to both receptor- and nonreceptor-type protein-tyrosine kinases. These enzymes include PtdIns 3-kinase, PtdIns-specific PLC- γ , ras GAP, and the pp74^{c-raf} serine/threonine kinase. Table 1 summarizes the evidence that these enzymes are targets of various protein-tyrosine kinases.

PtdIns 3-Kinase Association with pp60^{c-src}

PtdIns 3-kinase was the first of this group of proteins to be found associated with a tyrosine kinase. Until recently, phosphatidylinositol was thought to be phosphorylated only at the D-4 and D-5 positions of the inositol ring (for review see Carpenter and Cantley, 1990). The discovery of PtdIns 3-kinase uncovered a family of D-3 phosphorylated phosphoinositides that appear to act in a new signaling pathway distinct from the canonical PtdIns turnover pathway (Aüger et al., 1989b; Whitman et al., 1988).

PtdIns, PtdIns 4-phosphate, and diacylglycerol have all been shown to be phosphorylated by activities that copurified with pp60^{c-src} (Sugimoto et al., 1984). Immunoprecipitates of pp68^{v-ros} were also found to contain PtdIns kinase activities (Macara et al., 1984). These activities did not easily separate from the protein-tyrosine kinase activity of pp60^{c-src} and pp68^{v-ros} and were inhibited in parallel

Table 1. Enzymes Directly Affected In Vivo by Activated Protein-Tyrosine Kinases

Tyrosine Kinase	PtdIns 3-Kinase	<i>ras</i> GAP	PtdIns- Specific PLC- γ	c-Raf Serine Kinase	Others Associated	References
Nonreceptor Family						
pp60 ^{v-src}	A, P-Y, †	P-Y	?	†		Ellis et al., 1990; Han et al., 1990; Morrison et al., 1988; Sugimoto et al., 1984
Middle t-pp60 ^{c-src}	A, P-Y, †	?	?	†	Phosphatase 2A	Morrison et al., 1988; Pallas et al., 1990; Whitman et al., 1985
Middle t-pp62 ^{c-yes}	A	?	?	?		Fukui et al., 1989
pp160 ^{gag-abl}	A, †	P-Y	?	?		Ellis et al., 1990; Varticovski et al., 1991
pp130 ^{gag-fps}	A	P-Y	?	?		Ellis et al., 1990; Fukui et al., 1989
pp59 ^{c-hyn} (thrombin-stimulated platelets)	A, †	?	?	?		Gutkind et al., 1990
Receptor Family						
PDGF receptor- β	A, P-Y, †	A, P-Y	A, P-Y, †	A, †	pp60 ^{c-src} , pp59 ^{c-hyn} , pp62 ^{c-yes}	Kaplan et al., 1987; Molloy et al., 1989; Kaplan et al., 1990; Whitman et al., 1987; Kaslauskas and Cooper, 1989; Wahl et al., 1989; Kaslauskas et al., 1990; Kypta et al., 1990; Morrison et al., 1989; Morrison et al., 1988; Morrison et al., 1990
CSF-1 receptor pp150 ^{c-fms}	A, †	A, P-Y	—	†		Varticovski et al., 1989; Reedijk et al., 1990; Roussel et al., 1990; Shurtleff et al., 1990; Baccarini et al., 1990; Choudhury et al., 1990
pp150 ^{v-fms}	A	P-Y	?	†		Ellis et al., 1990; Kaplan et al., 1987; Morrison et al., 1988; Varticovski et al., 1989
EGF receptor	±, A	P-Y	±, A, P-Y, †	?	PtdIns(4)P 5-kinase, PtdIns 4-kinase	Ellis et al., 1990; Margolis et al., 1989; Wahl et al., 1988; Bjorge et al., 1990; Pignataro and Ascoli, 1990; Cochet et al., 1991
Insulin receptor	A, †	?	—	†		Blackshear et al., 1990; Endemann et al., 1990; Kovacina et al., 1990; Ruderman et al., 1990
IGF-1 receptor pp68 ^{gag-ros}	A, † A	? ?	? ?	? ?		Kapeller et al., submitted Fukui et al., 1989; Macara et al., 1984

Symbols: A, direct physical association with protein-tyrosine kinase; P-Y, evidence that enzyme is phosphorylated on tyrosine in vivo; †, evidence either that the specific activity of the enzyme increases in vitro or products of the enzyme are elevated in vivo; ±, evidence for association in certain cell types but not in others.

with the tyrosine kinase activity; for these reasons they were originally thought to be intrinsic activities of the same polypeptide. The PtdIns kinase activity was also found to coimmunoprecipitate with the polyoma middle t-pp60^{c-src} complex of polyoma-infected cells (Whitman et al., 1985). The PtdIns kinase activity was shown to be separable from the middle t-pp60^{c-src} complex in very high salt and detergent solutions (Kaplan et al., 1986; Whitman et al., 1987). This enzyme was then shown to be distinct from the major PtdIns 4-kinase activity in fibroblasts (Whitman et al., 1987) and to phosphorylate the D-3 position of the inositol ring (Whitman et al., 1988).

Interest in the PtdIns 3-kinase was piqued before the

discovery that it functioned in a new pathway because association of this enzyme with polyoma middle t tightly correlated with cell transformation. All mutants of middle t that failed to associate with the PtdIns kinase failed to transform 3T3 fibroblasts (Courtneidge and Heber, 1987; Kaplan et al., 1986, 1987; Whitman et al., 1985). Subsequent studies indicated that mutants of pp60^{v-src} (Fukui and Hanafusa, 1989) and of pp130^{gag-abl} (Varticovski et al., 1991) that failed to associate with PtdIns 3-kinase failed to transform cells. Mutations in pp60^{c-src} that caused transformation caused association with PtdIns 3-kinase (Chan et al., 1990).

For most of the oncogene mutants investigated, the abil-

ity of the gene product to associate with PtdIns 3-kinase correlated with the level of protein-tyrosine kinase activity, which also correlated with the ability to transform fibroblasts. However, a family of middle t mutants that associate with pp60^{c-src} and activate tyrosine kinase activity but fail to transform cells in culture have been described (Courtneidge and Heber, 1987; Druker et al., 1990; Kaplan et al., 1986; Whitman et al., 1985). Some of these mutants have been found to be defective in association with the PtdIns 3-kinase or activation of this enzyme in vivo, indicating an essential role of this enzyme in transformation by middle t (Whitman et al., 1985; Kaplan et al., 1986; Serunian et al., 1990; Courtneidge and Heber, 1987). The most interesting of this group of mutants is a Tyr→Phe substitution at position 315, the major site of tyrosine phosphorylation in vivo (Whitman et al., 1985). Later studies strongly implicated this region of middle t in binding of PtdIns 3-kinase and in the ability of polyomavirus to induce tumors in vivo (Talmage et al., 1989).

An 85 kd phosphoprotein was implicated as a subunit of the PtdIns 3-kinase. This protein was shown to correlate with the appearance of this activity in immunoprecipitates of a number of middle t mutants (Courtneidge and Heber, 1987; Kaplan et al., 1987). The same protein was also shown to appear in parallel with PtdIns 3-kinase activity in anti-phosphotyrosine immunoprecipitates of PDGF-stimulated cells and to copurify with the PDGF receptor (Kaplan et al., 1987). Phosphoamino acid analysis revealed that the 85 kd protein was phosphorylated on both serine and tyrosine residues in middle t-transformed cells and PDGF-stimulated cells (Kaplan et al., 1987). This protein was also shown to be phosphorylated by the tyrosine kinase activities of these complexes following immunoprecipitation, indicating that the 85 kd subunit of PtdIns 3-kinase is a direct substrate of pp60^{c-src} and the PDGF receptor (Kaplan et al., 1987). The association of this enzyme with the PDGF receptor is discussed further below.

The PtdIns 3-kinase is a tightly associated heterodimer of the 85 kd protein and a previously undescribed 110 kd protein (Carpenter et al., 1990). The purified PtdIns 3-kinase tightly associates with tyrosine-phosphorylated middle t in the absence of pp60^{c-src} or with tyrosine kinase-active pp60^{v-src} in the absence of middle t, apparently through distinct domains. At least for polyoma middle t, this interaction occurs through the 85 kd subunit (Carpenter et al., 1990; Cohen et al., 1990a, 1990b). The region of middle t involved in this association is apparently around Tyr-315. As mentioned above, this is the major site of phosphorylation of middle t by pp60^{c-src}, and mutation of this residue to a Phe reduces (but does not eliminate) association with PtdIns 3-kinase (Whitman et al., 1985; Talmage et al., 1989). The domain of p85 involved in the association with middle t has not been determined. However, cDNA clones of the 85 kd subunit of PtdIns 3-kinase were recently sequenced and determined to have two SH-2 domains and an SH-3 domain (M. Waterfield and P. Parker, personal communication; L. Williams, personal communication). Thus, the prediction of the model in Figure 3 is that an SH-2 domain of the 85 kd subunit of PtdIns 3-kinase associates with the phosphotyrosine-315 region of middle t.

The mechanism by which pp60^{v-src} associate PtdIns 3-kinase is likely to involve the SH-2 domain of pp60^{v-src} and a tyrosine-phosphorylated region of PtdIns 3-kinase (Figure 3). As discussed above, amino acid substitutions in the SH-2 domain of pp60^{v-src} increase PtdIns 3-kinase association and increase transformation without causing dephosphorylation of PtdIns 3-kinase at Tyr-527 or increased phosphorylation at Tyr-416 (O'Brien et al., 1990).

A significant number of protein-tyrosine kinases, Src-like and receptor-type, have now been shown to associate with PtdIns 3-kinase (Table 1). In all cases investigated, the association has been found to be dependent on the activation of protein-tyrosine kinase activity, by addition of growth factors or hormones or by mutation. Association of PtdIns 3-kinase with activated protein-tyrosine kinases appears to be almost ubiquitous (see discussion of receptor-type tyrosine kinases). Thus, it is likely that the primordial protein-tyrosine kinase with which these proteins evolved associated with the PtdIns 3-kinase and this association has been maintained throughout evolution. Significantly, both tyrosine kinase activity (Gould and Nurse, 1989) and PtdIns 3-kinase activity (Auger et al., 1989a) exist in eukaryotic systems from man to yeast.

ras GAP Is Tyrosine-Phosphorylated in Cells Transformed by both Receptor- and Nonreceptor-Type Protein-Tyrosine Kinases

ras GAP has been shown to be phosphorylated on tyrosine in cells transformed by several oncogenes (Ellis et al., 1990). As discussed in more detail below, *ras GAP* is also phosphorylated in response to stimulation of cells with PDGF, epidermal growth factor (EGF), or c-fos stimulating factor 1 (CSF-1) (Ellis et al., 1990; Kaplan et al., 1990; Kazlauskas et al., 1990; Molloy et al., 1989; Reilly et al., 1990). Unidentified 62 kd and 190 kd protein kinases associate with *ras GAP* in cells transformed by protein-tyrosine kinase type oncogenes. These proteins are phosphorylated on tyrosine (the obvious candidate for these proteins, pp60^{c-src} and the PDGF receptor, have been eliminated [Kazlauskas et al., 1990; Polakis et al., 1990]). Thus, when certain cellular protein-tyrosine kinases are activated, *ras GAP* becomes phosphorylated on tyrosine and tightly associates with other tyrosine-phosphorylated proteins.

The mechanism by which *ras GAP* complexes with other tyrosine-phosphorylated proteins is also likely to involve formation of an SH-2 domain-phosphotyrosine complex (see Figure 3). *ras GAP* has two SH-2 domains and one of these is required for association with *ras* (Polakis et al., 1990), although neither is needed for GTPase stimulating activity. Potentially the tyrosine-phosphorylated region of *ras GAP* could be involved in forming associations with other proteins containing SH-2 domains. The role of *ras GAP* in tyrosine kinase signaling is discussed further below.

Raf Activation By Protein-Tyrosine Kinases

The protein-serine/threonine kinase pp74^{c-raf} can be immunoprecipitated by an anti-phosphotyrosine antibody from cells transformed by *src*-like oncogenes (Morrisson et al., 1988; reviewed by Roberts, 1991). The immunoprecipitation

tated pp74^{c-raf} had an altered mobility on SDS gel electrophoresis and had enhanced phosphorylation on serine. It was also more active than unphosphorylated pp74^{c-raf}, indicating that the tyrosine kinases were somehow activating the serine kinase activity of pp74^{c-raf}. This activation was a key discovery, since it provided a link between the tyrosine kinase family of oncogenes and a proto-oncogene of the serine kinase family.

Previous studies suggested that v-*raf* transformed by activating events downstream of tyrosine kinases and *ras* (Smith et al., 1986). Although pp74^{c-raf} is immunoprecipitated by anti-phosphotyrosine antibodies from v-*src*-transformed cells, most of the increase in phosphorylation appears to be on serine residues. Thus, pp74^{c-raf} differs from PtdIns 3-kinase and *ras* GAP in that it is weakly phosphorylated on tyrosine. Significantly, pp74^{c-raf} lacks SH-2 domains, so its recruitment to activated protein-tyrosine kinases does not appear to be explained by the model in Figure 4. Possibly pp74^{c-raf} is associated with another protein that contains SH-2 domains and that becomes phosphorylated on tyrosine. The possible signaling pathway for pp74^{c-raf} is discussed below.

Other Targets of Nonreceptor Protein-Tyrosine Kinases

A number of other proteins have been shown to become phosphorylated on tyrosine in cells transformed by *Src* and related oncogenes. These include the lipocortin/calpactin family of proteins (Pepinsky and Sinclair, 1986; Saris et al., 1986), ezrin (Gould et al., 1989), a 42 kd serine/threonine kinase (Ely et al., 1990; Kazlauskas and Cooper, 1988), and a host of others (reviewed by Hunter et al., 1988). The lipocortin/calpactin proteins undergo calcium-dependent association with membranes, and one protein in this family has been implicated as an inositol 1,2-cyclic monophosphate phosphohydrolase (Ross et al., 1990). They do not have SH-2 domains and, unlike the group of proteins discussed above, they do not appear to associate tightly with protein-tyrosine kinases. In many cells these are the most easily detected tyrosine-phosphorylated proteins, but their phosphorylation has not correlated well with cell transformation. Ezrin has structural homology with a domain of the red cell actin-binding protein band 4.1 that is not part of the actin-binding domain (Gould et al., 1989). The effect of activation of protein-tyrosine kinases on actin rearrangement is discussed further below.

Phosphorylation of the p42 protein has correlated well with cell transformation and mitogenesis. This protein appears to be a serine/threonine kinase that phosphorylates several interesting proteins in vitro, including one of the ribosomal S6 kinases and a microtubule-associated protein, MAP-2 (Ely et al., 1990; Sturgill et al., 1988). The activity of this enzyme appears to be completely dependent on phosphorylation at both threonine and tyrosine residues (Anderson et al., 1990; Meier et al., 1990). Thus, this protein may be in the middle of a kinase cascade that begins with activation of plasma membrane protein-tyrosine kinases and ends with phosphorylation of the ribosomal subunit S6. Phosphorylation of S6 occurs in re-

sponse to a wide variety of growth factors and hormones and probably has a regulatory effect on protein synthesis (for review see Ballou et al., 1988).

It is not clear which protein-tyrosine kinase phosphorylates p42 in vivo. In vitro, this protein is not a good substrate for the *Src*-type protein kinases or the insulin receptor. Activation of PKC with phorbol esters can somehow stimulate phosphorylation of p42 on tyrosine (Kazlauskas and Cooper, 1988). Down-regulation of PKC reduces PDGF and EGF induction of p42 phosphorylation. Thus, phosphorylation of this protein may be several steps downstream of the primary targets of pp60^{src} and of the receptor-type protein-tyrosine kinases.

Implications of Membrane Localization of pp60^{src} and Its Targets

As discussed above, mutations that prevent amino-terminal myristoylation of pp60^{v-src} and other *Src*-like oncoproteins prevent transformation. This result has been interpreted as evidence that a critical substrate of the tyrosine kinase activity is at the plasma membrane. However, in view of the model in Figure 3, an alternative hypothesis is suggested: pp60^{c-src} could act to recruit enzymes to critical locations in cell membranes. For example, PtdIns 3-kinase phosphorylates membrane lipids and must therefore be recruited to the membrane to interact with its substrates. Thus, it is possible that PtdIns 3-kinase and other enzyme substrates of pp60^{src} perform their functions only while complexed with pp60^{src}. Mutational studies of the *c-abl* gene product support this idea (Varticovski et al., 1991). Like v-*Src* mutants, v-*Abl* mutants lacking the amino-terminal myristoylation sequence fail to transform fibroblasts, even though they phosphorylate cellular proteins on tyrosine. Immunoprecipitates of the mutant *Abl* protein have levels of PtdIns 3-kinase activity similar to those found in immunoprecipitates of the transforming gene product. However, a product of this enzyme, PtdIns(3,4)P₂, is not significantly elevated compared with levels in the transformed cells. Thus, the failure of the *Abl*-PtdIns 3-kinase complex to associate with the membrane where the substrates of PtdIns 3-kinase reside may explain the reduced level of products and the nontransforming phenotype.

A nontransforming mutation (Pro-248→Leu) of the polyoma middle t gene suggests a more subtle aspect of membrane localization of the middle t-pp60^{c-src} complex necessary for transformation. This mutant middle t protein associates with the membrane, complexes with pp60^{c-src}, stimulates the pp60^{c-src} protein-tyrosine kinase activity, becomes phosphorylated on tyrosine, and complexes with PtdIns 3-kinase much like wild-type middle t (Druker et al., 1990). However, this mutant failed to cause transformation of fibroblasts in culture. Interestingly, the sequence surrounding Pro-248 satisfies the rules for a coated vesicle localization signal determined in the low density lipoprotein receptor (Chen et al., 1990). This observation raises the exciting possibility that the middle t-pp60^{c-src}-PtdIns 3-kinase complex may associate with a coated pit or be internalized to produce a transforming signal.

Growth Factor Receptor Protein-Tyrosine Kinases: PDGF Receptor as the Prototype

Protein-tyrosine kinases of the receptor family have a cytosolic domain with sequence homology to the kinase domain of pp60^{c-src} but otherwise differ in structure. They have an extracellular ligand-binding domain, a single transmembrane domain, and lack the SH-2 and SH-3 domains common to the nonreceptor family. The protein-tyrosine kinase activity of these receptors is regulated by ligand binding at the extracellular domain (reviewed by Ullrich and Schlessinger, 1990; Yarden and Ullrich, 1988). The prevailing evidence suggests that ligand binding allows adjacent cytosolic domains within a receptor dimer to cross-phosphorylate each other on tyrosine residues, thereby causing a conformational change that enhances kinase activity toward other substrates. Oncogenic variants of these receptors arise as a result of mutations that allow constitutive protein-tyrosine kinase activity in the absence of ligand. Activating mutations that have been described for receptor-like kinases include deletion of large portions of the extracellular domain, single amino acid substitutions in the transmembrane domain, and mutations in the cytosolic domain (reviewed in Ullrich and Schlessinger, 1990; Yarden and Ullrich, 1988). It appears likely that most of these mutations enhance ligand-independent dimerization and cross-phosphorylation.

Alternatively, oncogenesis can occur by simultaneous expression of receptors and activating ligands in the same cell (autocrine stimulation). The classic example of this mechanism is the *v-src* oncogene, which encodes a homolog of the PDGF-B chain and transforms fibroblasts by activating the endogenous PDGF receptor (Waterfield et al., 1983). Autocrine stimulation is discussed in more detail by Cross and Dexter (1991 [this issue of *Cell*]).

PDGF-Dependent Recruitment of Cytosolic Enzymes to the PDGF Receptor

The mechanism by which tyrosine kinase-type receptors stimulate secondary responses has become clearer in the past 2 years. The ligand-stimulated autophosphorylation (or cross-phosphorylation) on tyrosine not only enhances the tyrosine kinase activity of the receptors but also creates binding sites for recruitment of specific enzymes. These enzymes then transduce signals to the cell interior that cause a variety of responses needed for a cell division cycle. The recruitment mechanism appears to utilize the SH-2 domain-phosphotyrosine association discussed above. Also, not surprisingly, some of the same proteins that are targets for the Src-like tyrosine kinases are recruited to the receptor-type tyrosine kinases.

The first evidence for ligand-dependent recruitment of enzymes to a tyrosine kinase-type receptor was the discovery that the PtdIns 3-kinase (a cytosolic enzyme in resting cells) is recruited to the PDGF receptor within less than 1 min following stimulation with PDGF (Kaplan et al., 1987; Whitman et al., 1987). This association was shown to require the "kinase-insert" domain (a region of se-

quence unique to the PDGF receptor that lies in the middle of the kinase domain; Figure 1) (Coughlin et al., 1989). A tyrosine residue in this domain (position 751 of the human PDGF β -receptor) undergoes autophosphorylation in response to addition of PDGF, and mutation of this residue to a phenylalanine dramatically reduces (but does not eliminate) the PtdIns 3-kinase recruitment (Kazlauskas and Cooper, 1989). Kinase-defective forms of the PDGF receptor completely fail to associate with PtdIns 3-kinase (Coughlin et al., 1989; Kazlauskas and Cooper, 1989). A synthetic peptide based on this region of the PDGF receptor and containing phosphate at the tyrosine equivalent to Tyr-751 blocked association of the PDGF receptor with PtdIns 3-kinase (Escobedo et al., 1991). The same peptide phosphorylated at Tyr-740 also blocked association. The unphosphorylated peptide was ineffective. The sequence immediately around Tyr-751 of the PDGF receptor has similarity with the sequence around Tyr-315 of polyoma middle T (the region implicated in PtdIns 3-kinase association; see Figure 3). The sequence around Tyr-740 also shows homology, and it is likely that the conserved residues provide an optimal binding site for an SH-2 domain of the 85 kd subunit of the PtdIns 3-kinase (see model in Figure 4). A possible consensus sequence for PtdIns 3-kinase binding is presented in Figure 5.

As mentioned above, the PDGF receptor was recently shown to undergo ligand-dependent association with *ras* GAP (Kaplan et al., 1990; Kazlauskas et al., 1990; Molloy et al., 1989). The model in Figure 4 suggests that this association also occurs via an SH-2 domain of *ras* GAP binding to a tyrosine-phosphorylated domain of the receptor. The domain involved is not completely clear, but phosphorylation of Tyr-751 is not primarily responsible for this association (Kazlauskas et al., 1990).

PtdIns-specific PLC- γ was also shown to undergo ligand-dependent association with both EGF and PDGF receptors (Margolis et al., 1989; Meisenhelder et al., 1989; Morrison et al., 1990; Wahl et al., 1988, 1989). This enzyme also has SH-2 domains (Stahl et al., 1988; Suh et al., 1988b) and probably associates by a mechanism similar to that proposed for PtdIns 3-kinase and *ras* GAP (Figure 4). Deletion of the kinase-insert domain or mutation of Tyr-751 does not affect PtdIns-specific PLC- γ association with the PDGF receptor, indicating that it binds to a domain distinct from that which binds PtdIns 3-kinase (Coughlin et al., 1989; Kazlauskas and Cooper, 1989; Morrison et al., 1990). These results suggest that the SH-2 domains of these three targets of the PDGF receptor have preferential affinities for distinct tyrosine-phosphorylated domains of the receptor (Figure 4).

The PDGF receptor also undergoes ligand-dependent association with pp74^{c-raf} (Morrison et al., 1988, 1989). This association also appears to require autophosphorylation of the receptor on tyrosine, although additional work is necessary to determine the specific domains involved in association. Unlike the other three targets, pp74^{c-raf} does not have an SH-2 domain. The mechanism of its association is unknown.

PROTEIN	SEQUENCE	PHOSPHORYLATED IN VIVO
POLYOMA MIDDLE T (TYR315)	E E E E E Y M P M E D	YES
HUMAN PDGF R- β (TYR751)	D E S V D Y V P M L D	YES
MOUSE PDGF R- β (TYR750)	D E S I D Y V P M L D	?
HUMAN PDGF R- α (TYR742)	A D T T Q Y V P M L E	?
HUMAN PDGF R- β (TYR740)	E S D G G Y M D M S K	?
MOUSE PDGF R- β (TYR739)	E S D G G Y M D M S K	?
HUMAN PDGF R- α (TYR731)	E N N G D Y M D M K Q	?
HUMAN C-KIT (TYR721)	D S T N E Y M D M K Q	?
HUMAN CSF-1 R (TYR721)	Q G V D T Y V E M R P	?
HUMAN MET (TYR1331)	C P D P L Y E V M L K	?
MOUSE MET (TYR1311)	C P D A L Y E V M L K	?
HUMAN INSULIN R (TYR1322)	E E H I P Y T H M N G	YES
HUMAN IGF-1 R (TYR1346)	D E R Q P Y A H M N G	?
RAT IGF-1 R (TYR1362)	D E H I P Y T H M N G	?
CONSENSUS SEQUENCE:	E E E E E Y M P M X X	
	D D D D D Y V P M X X	

where X is a hydrophilic amino acid.

Figure 5. A Consensus Sequence for Protein-Tyrosine Kinase Auto-phosphorylation Sites That Bind PtdIns 3-Kinase

Phosphorylation of Tyr-315 of polyoma middle t and phosphorylation of Tyr-751 of the human PDGF β -receptor have been shown to affect association of the respective proteins with the PtdIns 3-kinase (see text). In the mouse PDGF β -receptor both Tyr-750 (equivalent to Tyr-751 of human) and Tyr-739 have been implicated as binding sites for PtdIns 3-kinase. Regions with sequence similarity are found in other receptors known to bind PtdIns 3-kinase (see Table 1). In a few cases these sites are known to be phosphorylated in vivo. For the PDGF α and β receptors, c-Kit, and CSF-1 receptors, the sequences are located in the kinase-insert domains. For the insulin receptor, IGF-1 receptor, and c-Met protein, the sites are in the carboxy-terminal nonkinase domain. Consistent with its relatively low affinity for the PtdIns 3-kinase, the insulin receptor has a sequence with relatively weak similarity. This site is known to be phosphorylated in vivo. A highly conserved region of the tyrosine kinase domain (ATSEVY₉₂₆EIMVK in the human PDGF β -receptor) has some similarity to the consensus sequence but appears not to be involved in PDGF receptor association with PtdIns 3-kinase. The only region of the human EGF receptor with significant homology to the consensus sequence is CTIDVY₉₂₀MIMVK in the conserved tyrosine kinase domain. There are no reports of tyrosine phosphorylation in this region of the EGF receptor or PDGF receptor.

Role of Phosphorylation in Activation of PtdIns 3-kinase, *ras* GAP, PtdIns-Specific PLC- γ , and pp74^{c-raf}

The possibility that association with the PDGF receptor alters the activities of these enzymes is under investigation. All four of these proteins undergo a change in phosphorylation upon binding to the PDGF receptor. In the cases of PtdIns 3-kinase (Kaplan et al., 1987), PtdIns-specific PLC- γ (Meisenhelder et al., 1989; Morrison et al., 1990; Wahl et al., 1989), and *ras* GAP (Kaplan et al., 1990; Kazlauskas et al., 1990; Molloy et al., 1989), an increase

in tyrosine phosphorylation occurs. The pp74^{c-raf} protein is mostly phosphorylated on serine (although some tyrosine phosphorylation may occur in some cells). The 85 kd subunit of PtdIns 3-kinase also appears to undergo an increase in serine phosphorylation in response to PDGF (Kaplan et al., 1987). The highly phosphorylated pp74^{c-raf} has increased protein-Ser/Thr kinase activity, and in vitro phosphorylation of pp74^{c-raf} on tyrosine enhances its serine kinase activity (Morrison et al., 1988, 1989). A variety of stimuli, including insulin (Blackshear et al., 1990; Kovacina et al., 1990) and phorbol esters, can cause an increase in serine phosphorylation of pp74^{c-raf}.

Phosphorylation of PtdIns-specific PLC- γ on tyrosine by the EGF receptor appears to increase its catalytic activity. Immunoprecipitates of PtdIns-specific PLC- γ 1 from A-431 cells can be phosphorylated by purified EGF receptor, and this results in a 4-fold increase in catalytic activity (Nishibe et al., 1990). Other recent experiments suggest an additional complexity to the mechanism for regulation of PtdIns-specific PLC- γ via tyrosine phosphorylation. Purified PtdIns-specific PLCs have relatively high specific activities in vitro. However, addition of physiologically relevant levels of profilin (an actin-binding protein) dramatically inhibits the PtdIns-specific PLC hydrolysis of PtdIns(4,5)P₂ (Goldschmidt-Clermont et al., 1990). This inhibition is apparently due to the ability of profilin to associate tightly with PtdIns(4,5)P₂. Recently it has been shown that treatment of purified PtdIns-specific PLC- γ with EGF plus EGF receptor plus ATP activates the PtdIns-specific PLC- γ by relieving the profilin inhibition (Goldschmidt-Clermont et al., J. Cell Biol. 111, abstract 455, 1990). Thus, tyrosine phosphorylation of PtdIns PLC- γ may increase its activity by increasing its affinity for substrates or by allowing it to utilize lipid that is bound to profilin. This result suggests a very interesting link between PtdIns turnover and actin-binding proteins. The phosphoinositide/actin-binding protein connection is discussed further below.

Thus far there is no conclusive evidence that phosphorylation on tyrosine changes the activity of PtdIns 3-kinase or *ras* GAP. Notably, PtdIns 3-kinase, PtdIns-specific PLC- γ , and *ras* GAP all have substrates that reside at the plasma membrane. Conceivably, recruitment to the membrane alone could contribute to activation of these enzymes. The PDGF receptor could merely bring these enzymes to the proper location for substrate utilization without dramatically affecting the intrinsic enzymatic activity. However, in view of the evidence for stoichiometric phosphorylation of the receptor-bound forms of these proteins, it is likely that tyrosine phosphorylation is important for activity.

Targets of Other Receptor-Type Protein-Tyrosine Kinases

Other receptor-type protein tyrosine kinases that have been investigated associate with a subgroup of the enzymes that bind to the PDGF receptor (Table 1). Interestingly, none of the other receptors thus far investigated appear to associate with all four of the enzymes discussed above—at least, not as efficiently as does the PDGF receptor. In addition, each of the receptors investigated

appears to have specificity for a distinct subgroup of these four targets.

The CSF-1 receptor (*c-fms* product) associates with PtdIns 3-kinase (Varticovski et al., 1989) and *ras* GAP (Reedijk et al., 1990) but appears not to associate with PtdIns-specific PLC- γ . A consensus sequence for binding PtdIns 3-kinase is found in the kinase-insert domain of *c-Fms* (Figure 5), but this site has not been shown to be phosphorylated. Deletion of the kinase-insert domain reduced (but did not eliminate) PtdIns 3-kinase association (Reedijk et al., 1990; Shurtleff et al., 1990). The kinase-insert deletion mutant was impaired in stimulation of growth in some cells but not others (Reedijk et al., 1990; Shurtleff et al., 1990). The question of pp74^{c-*raf*} association with the *c-Fms* protein is being investigated, but transformation with *v-fms* alters the phosphorylation state of pp74^{c-*raf*} (Morrison et al., 1988).

As discussed above, the EGF receptor appears to associate with PtdIns 3-kinase in some cells (Bjorge et al., 1990). The ability of EGF to activate PtdIns-specific PLC- γ requires that a relatively large number of receptors be present (Margolis et al., 1989; Wahl et al., 1988). Some of the diversity in EGF responses in different cells might be explained by the recent finding that EGF induces formation of a heterodimer of the EGF receptor (*c-ErbB*) and the *c-ErbB2* protein in cells that express both proteins (Wada et al., 1990). Thus, it is likely that the EGF receptor-*ErbB2* complex stimulates different responses than does the EGF receptor homodimer (Di Fiore et al., 1990). In any event, the PDGF receptor appears to have a consistently greater effect on PtdIns 3-kinase than does the EGF receptor (Kaplan et al., 1987). Consistent with this finding, the EGF receptor does not have a good consensus sequence for PtdIns 3-kinase binding (Figure 5). The EGF receptor also associates with *ras* GAP (Ellis et al., 1990). In addition, PtdIns(4)P 5-kinase and PtdIns 4-kinase (enzymes in the canonical PtdIns turnover pathway) associate with the EGF receptor (Cochet et al., 1991). Thus, in normal tissues this receptor probably stimulates *ras* GAP, PtdIns 4-kinase, PtdIns(4)P 5-kinase, and in some instances PtdIns-specific PLC- γ and PtdIns 3-kinase. The consequence is likely to be very different from PDGF stimulation.

The discovery that the insulin receptor associates with the PtdIns 3-kinase and activates production of the D-3 phosphoinositides provides the first evidence for a direct target of this receptor that causes a physiological response. Insulin induces a variety of cellular responses, including changes in tyrosine phosphorylation of several proteins. However, until recently it has been difficult to determine the function of these proteins or to prove that they are direct substrates of the insulin receptor. A fraction of the cellular PtdIns 3-kinase coimmunoprecipitates with the activated insulin receptor, providing strong evidence for a direct effect on this enzyme (Ruderman et al., 1990). However, most of the anti-phosphotyrosine antibody precipitated PtdIns 3-kinase from insulin-stimulated cells is not associated with the insulin receptor (Endemann et al., 1990; Ruderman et al., 1990). Thus, unlike the PDGF receptor, which appears to retain most of the tyrosine-

phosphorylated PtdIns 3-kinase, the insulin receptor releases this enzyme more readily. The insulin receptor appears not to associate with or phosphorylate PtdIns-specific PLC- γ , but it does cause activation of pp74^{c-*raf*}, apparently by causing an increase in serine phosphorylation (Blackshear et al., 1990; Kovacina et al., 1990). No effect of insulin receptor on *ras* GAP has thus far been reported. Thus, the insulin receptor activates PtdIns 3-kinase and pp74^{c-*raf*} but apparently does not affect PtdIns-specific PLC- γ .

All the other receptor-type tyrosine kinases that have been investigated appear to associate with PtdIns 3-kinase when activated (Table 1). These include insulin-like growth factor 1 (IGF-1) receptor, pp190^{c-*met*}, pp68^{gag-*ros*}, and pp145^{v-*kit*}. Further work is needed to determine which other proteins associate with these activated protein-tyrosine kinases.

The Role of Primary Targets of Growth Factor Receptor Tyrosine Kinases in the Growth Response

The pattern emerging in Table 1 suggests a possible explanation for the necessity of multiple growth factors for optimal cell division. Of the receptors listed, only the PDGF receptor appears to be capable of mediating ligand-stimulated cell division in normal tissues in the absence of other growth factors. Signaling by PDGF is complicated by the existence of three forms of PDGF (BB, AA, and AB) and three types of PDGF receptor dimers ($\alpha\alpha$, $\beta\beta$, and $\alpha\beta$), each of which appears to provide distinct signals to the cell (Heldin and Westermark, 1990). The $\beta\beta$ -type receptor was the focus of most of the studies discussed above. Significantly, the PDGF receptor is the only receptor listed in Table 1 that is known to associate tightly with all four of the targets discussed above. Other growth factors can enhance the mitogenic effects of PDGF, suggesting that there are other important targets that PDGF fails to activate. Alternatively, one could argue that the inefficiency of PDGF as a mitogen acting alone is due to PDGF receptor down-regulation. The ability of *v-sis* (which encodes a homolog of PDGF BB) and the cellular gene encoding PDGF BB (Beckmann et al., 1988) to transform cells supports the idea that the PDGF receptor triggers the critical events for cell growth.

Thus, one could argue that turning on any combination of receptors that activate the same targets as the PDGF receptor should produce a mitogenic response. Insulin (or IGF-1) in combination with EGF should turn on the same pathways as PDGF (provided the receptors are in sufficient abundance in the cell; see Table 1). The predictive nature of this model is complicated by the possibility that one or more of the targets in Table 1 is spontaneously activated in a particular tissue or cell line, thereby rendering that cell line responsive to a single mitogen that activates the remaining targets. It is also likely that other targets of protein-tyrosine kinases critical for cell division are yet to be described.

necessity, Sufficiency, and Redundancy

The discussion above implies that no single target of the protein-tyrosine kinases is sufficient to mediate cell

growth or transformation. Mutational studies of the PDGF and CSF-1 receptors support this idea. The kinase-insert deletion, discussed above, eliminates association with PtdIns 3-kinase and renders the PDGF receptor incompetent for stimulation of DNA synthesis but does not affect stimulation of PtdIns turnover (Coughlin et al., 1989). A similar deletion of the kinase-insert of the CSF-1 receptor significantly reduces (but does not eliminate) the PtdIns 3-kinase association (Reedijk et al., 1990; Shurtleff et al., 1990) but does not affect association with or phosphorylation of *ras* GAP (Reedijk et al., 1990). This mutant receptor is still capable of conferring CSF-1-dependent transformation on certain cells but is defective in transforming other cells (Reedijk et al., 1990; Shurtleff et al., 1990). These results are similar to results with the Tyr-315→Phe mutant of polyoma middle t (discussed above), which has dramatically reduced association with PtdIns 3-kinase and is defective in transforming some cells but not others.

On the other hand, there is evidence that activation of PtdIns 3-kinase alone is also insufficient to stimulate cell growth or transformation. The Pro-248→Leu mutant of polyoma middle t associates with PtdIns 3-kinase but fails to transform cells (Druker et al., 1990) as discussed above. Also, insulin alone activates the PtdIns 3-kinase, but a second mitogen is usually required to stimulate cell growth (Endemann et al., 1990; Ruderman et al., 1990). A mutation at Tyr-809 of the CSF-1 receptor blocks transformation without affecting PtdIns 3-kinase association (Roussel et al., 1990). Similarly, various agents that stimulate the canonical PtdIns turnover pathway fail to stimulate growth when added alone to quiescent cells (for review see Whitman and Cantley, 1988). Also, insulin alone activates pp74^{c-raf} without causing a mitogenic response, indicating that this activation alone is insufficient to cause cell growth (Blackshear et al., 1990; Kovacina et al., 1990). Further work is needed to determine whether tyrosine phosphorylation of *ras* GAP is sufficient to cause growth or transformation.

One problem with this simple-minded approach to the sufficiency of a pathway for stimulation of cell growth is that the magnitude and duration of a particular response are certain to be critical for cell division. For example, PDGF must be continuously present for at least 1 hr before fibroblasts are competent to proliferate in response to a second growth factor. Some of the PDGF responses (e.g., PtdIns 3-kinase activation; Auger et al., 1989b) are retained throughout this period, and other responses (e.g., conversion of phosphatidylcholine to diacylglycerol; Larrodera et al., 1990) require at least this long to occur. Thus, the failure of insulin alone to act as a mitogen could be due to a relatively weak or transient activation of PtdIns 3-kinase and/or pp74^{c-raf}. Microinjection of a mutant form of c-Raf-1 causes DNA synthesis and cell transformation, indicating that this pathway alone, if sufficiently activated, accomplishes cell transformation (Smith et al., 1990). Similarly, although activation of PtdIns-specific PLC by growth factors appears to be neither necessary (see below) nor sufficient for a growth response, microinjection of large amounts of PtdIns-specific PLC into fibroblasts caused a transformed phenotype (Smith et al., 1989).

Another problem in ascertaining the importance of a particular signaling pathway is redundancy: multiple pathways for activating the same cellular event. For example, transcription of the proto-oncogenes *c-fos* and *c-myc* can be induced by activators of protein kinase C (PKC); however, these same messages can be induced by PKC-independent pathways (Berkowitz et al., 1989; Coughlin et al., 1985; Fisch et al., 1989; Fukumoto et al., 1990; Gilman, 1988; Kaibuchi et al., 1989; Mehmet et al., 1990; Riabowol et al., 1988; Stumpo et al., 1988). Down-regulation of PKC does not prevent induction of *c-myc* by PDGF or of *c-fos* by insulin (Coughlin et al., 1985; Stumpo et al., 1988). Similarly, phosphorylation of the ribosomal protein S6 occurs by both PKC-dependent and PKC-independent pathways (Šušar et al., 1989). Thus, although activation of pathways that turn on PKC can cause these responses, many receptors accomplish the same events without activation of PKC. Tyrosine kinase-type growth factor receptors use both PKC-dependent and PKC-independent pathways to induce *c-myc* and *c-fos* mRNA and S6 phosphorylation.

Because of the redundancy discussed above, it is possible for a pathway to be sufficient for a cellular response without being necessary. Thus, although the PtdIns turnover-PKC pathway contributes to PDGF responses, there is evidence that inhibition of PDGF stimulation of PtdIns-specific PLC does not affect PDGF-stimulated DNA synthesis (Hill et al., 1990). This might partly be explained by the ability of PDGF to produce diacylglycerol by activation of phosphatidylcholine breakdown (Larrodera et al., 1990). The question of whether the other known targets of the PDGF receptor (PtdIns 3-kinase, *ras* GAP, and pp74^{c-raf}) are each absolutely necessary for the mitogenic response cannot yet be definitively answered. Thus far, mutants of the PDGF receptor (Coughlin et al., 1989), CSF-1 receptor (Reedijk et al., 1990; Shurtleff et al., 1990), and insulin receptor (Kapeller et al., submitted) that have reduced association with PtdIns 3-kinase were found to be at least partially defective in their mitogenic response, implying that activation of this enzyme is necessary for DNA synthesis. As discussed above, all mutants of polyoma middle t, pp60^{v-src}, and pp160^{gag-abl} that failed to associate with PtdIns 3-kinase were transformation defective, again arguing for the necessity of this response. Of course, these correlations do not prove necessity, since the mutations could also affect association with other targets. Further work is necessary to establish whether association of the PDGF receptor with *ras* GAP and pp74^{c-raf} is necessary for the mitogenic response.

Secondary Responses: Can All Cellular Responses to PDGF Be Explained by Four Targets?

A cascade of events likely to occur when a growth factor such as PDGF stimulates a cell is presented in Figure 6. It appears that many of the cellular responses known to occur can be explained as secondary responses to activation of PtdIns 3-kinase, PtdIns-specific PLC- γ , pp74^{c-raf}, and *ras* GAP. For example, c-Raf-1 enhances *c-fos* transcription by a PKC-independent pathway (Jamal and Ziff, 1990; Kaibuchi et al., 1989), so activation of pp74^{c-raf} might explain the PKC-independent pathway for *c-fos* in-

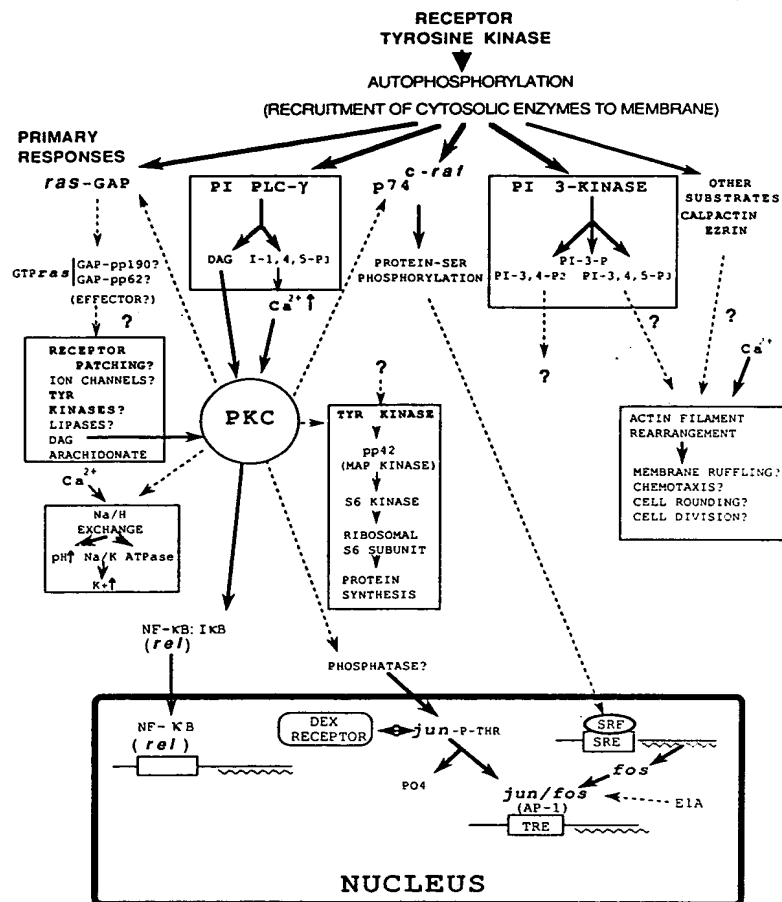


Figure 6. Secondary and Tertiary Responses to Activation of PtdIns-Specific PLC, PtdIns 3-Kinase, c-Raf Serine Kinase, and ras GAP

duction by PDGF, insulin, etc. Whether this is due to a direct phosphorylation of a component of the serum response factor remains to be seen. The increase in c-Fos protein could then produce a Jun-Fos complex (AP-1) capable of activating transcription of a variety of genes (reviewed by Curran and Franza, 1988). It is likely that other transcriptional cascades besides this one are triggered by Raf and are essential for growth. For a review of PDGF and serum-induced transcriptional cascades, see Bravo (1990).

Activation of PtdIns-specific PLC- γ would stimulate the canonical PtdIns turnover pathway, leading to elevation of cytosolic calcium and activation of PKC (reviewed by Whitman and Cantley, 1988). This pathway is known to activate a multitude of cellular events including changes in cytosolic pH, potassium level, and transcription of certain genes. One substrate of PKC is the inhibitory subunit (I κ B) of NF- κ B, phosphorylation of which releases NF- κ B and allows migration to the nucleus (Figure 6) (Ghosh and Baltimore, 1990). NF- κ B (the DNA-binding subunit of which is a member of the c-rel family; Ghosh et al., 1990; Kieran et al., 1990) binds to an element that controls expression of several genes in lymphocytes and other cells, including the κ light chain of immunoglobulin, IL-2, IL-2 receptor,

and β -interferon (reviewed in Lenardo and Baltimore, 1989). This is the first evidence of a direct mechanism for activation of transcription by PKC. Interestingly, the mRNAs of different members of the rel family can be induced by phorbol ester (apparently via activation of PKC; Bours et al., 1990; Bull et al., 1989). Activation of PKC also induces dephosphorylation of a critical threonine residue in the c-Jun protein that allows c-Jun to associate with the TPA response element (Boyle et al., 1991) and thereby activate transcription (Figure 6). The mechanism by which PKC causes dephosphorylation of c-Jun is unknown and could be very indirect.

Recently, a very exciting connection was made between PKC and p21^{c-ras}. Downward et al. (1990) showed that activation of the T cell antigen receptor results in conversion of p21^{c-ras} from the predominantly GDP-bound state to the GTP-bound state. The mechanism by which the T cell receptor accomplishes this activation is not completely clear, but PKC and ras GAP are involved. ras GAP from phorbol ester- or T antigen-stimulated cells had a reduced ability to stimulate GTP to GDP conversion on p21^{c-ras}, indicating that PKC activates Ras by preventing ras GAP from turning it off (Downward et al., 1990). However, PKC did not appear to phosphorylate ras GAP

directly. This discovery provides the first clear evidence for an activating pathway for p21^{c-ras} and argues that PKC functions upstream of Ras—at least in lymphocytes. In fibroblasts, the effects of phorbol esters or growth factors on the fraction of p21^{c-ras} in the GTP-bound state are subtle: PDGF causes a very small but detectable increase in GTP-p21^{c-ras} (Sato et al., 1990). However, there is evidence that PKC acts upstream of p21^{c-ras} in fibroblasts as well (Yu et al., 1988). It is possible that in fibroblasts Ras is activated by a recently discovered protein that stimulates GTP for GDP exchange (Wolfman and Macara, 1990).

The events triggered by activation of PtdIns 3-kinase are unknown. This enzyme converts the three phosphoinositides of the canonical PtdIns pathway (PtdIns, PtdIns(4)P, and PtdIns(4,5)P₂) to PtdIns(3)P, PtdIns(3,4)P₂, and PtdIns(3,4,5)P₃, respectively (reviewed by Carpenter and Cantley, 1990). The latter two lipids are undetectable in quiescent cells and appear in response to PDGF addition with a time course consistent with the recruitment of PtdIns 3-kinase to the PDGF receptor (Auger et al., 1989b). The time course for appearance and degradation of these lipids is consistent with them acting as second messengers rather than being precursors of a second messenger (although it is possible that they are both messengers and precursors of other messengers; see below). It is likely that PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ provide distinct signals to the cell interior since in smooth muscle and fibroblasts they appear to be made in parallel rather than as intermediates in a single pathway. Although the purified PtdIns 3-kinase efficiently converts PtdIns(4)P to PtdIns(3,4)P₂, in platelets a distinct pathway exists for synthesis of PtdIns(3,4)P₂: thrombin stimulates a recently discovered PtdIns(3)P 4-kinase that phosphorylates PtdIns(3)P at the 4 position (Yamamoto et al., 1991). Activation of this pathway results in a large production of PtdIns(3,4)P₂ but very little PtdIns(3,4,5)P₃. In smooth muscle and fibroblasts, where the PtdIns(3)P 4-kinase is undetectable, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ are produced in similar quantities in response to activation by PDGF, presumably by direct action of the PtdIns 3-kinase (Auger et al., 1989b). Thus, the levels of these two putative messengers can be independently controlled.

The phosphoinositides that are phosphorylated at the 3 position are not substrates for any of the known PtdIns-specific PLCs (Lips et al., 1989; Serunian et al., 1989), indicating that they are not converted to inositol polyphosphates in response to activators of the canonical pathway. One interesting possibility is that they are substrates for a phospholipase D. Hydrolysis of PtdIns(3,4,5)P₃ by a phospholipase D would produce inositol 3,4,5-trisphosphate, which could be converted to inositol 3,4,5,6-tetrakisphosphate. The level of an IP₄ of this apparent structure is elevated in v-src-transformed cells (Johnson et al., 1989). Inositol 3,4,5,6-tetrakisphosphate is thought to be an intermediate in synthesis of IP₅ and IP₆ (Stephens and Downes, 1990; Stephens and Irvine, 1990). Phospholipase D attack on PtdIns(3,4)P₂ would produce inositol 3,4-bisphosphate, which was recently found to be ele-

vated in polyoma middle t-transformed cells (Ulug et al., 1990).

One attractive possibility is that the polyphosphoinositides phosphorylated at the 3 position regulate actin filament rearrangements, as has been proposed for the canonical phosphoinositides. Actin filament rearrangement is a hallmark of growth factor stimulation and transformation and is probably at least partially responsible for membrane ruffling phenomena and cell rounding (Severinsson et al., 1990). In vitro, PtdIns(4,5)P₂ dissociates gelsolin from actin filaments (Janmey and Stossel, 1989) and dissociates profilin from actin monomers (Lassing and Lindberg, 1985). In fact, much of the PtdIns(4,5)P₂ in cells may be bound to profilin (Goldschmidt-Clermont et al., 1990). Other actin-binding proteins such as myosin type I (Adams and Pollard, 1989) and the glycophorin-band 4.1 complex (Anderson and Marchesi, 1985) also associate with PtdIns(4,5)P₂. In addition, casein kinase (type 1) tightly associates with this complex and is inhibited by the PtdIns(4,5)P₂ (Bazenet et al., 1990).

One problem with the model of PtdIns(4,5)P₂ acting as a regulator is that its total concentration in cells usually changes less than 2-fold in response to hormones or growth factors; however, large local changes are likely and can rescue the model. On the other hand, the total concentrations of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ change dramatically upon cell stimulation since these lipids are nominally absent in quiescent cells. The possibility that these lipids affect actin-binding proteins is under investigation. Since they are far less abundant than PtdIns(4,5)P₂, their cellular receptors must have very high affinity and specificity in binding. Although there is no convincing evidence thus far that the PtdIns 3-kinase affects cell morphology, it is interesting that a kinase-insert deletion mutant of the PDGF receptor (which should lack PtdIns 3-kinase association; Coughlin et al., 1989) still stimulates c-fos expression but fails to stimulate actin rearrangement (Severinsson et al., 1990).

The relevance of recruitment and tyrosine phosphorylation of ras GAP is unclear. One possibility is that this modification activates the Ras pathway by blocking the ability of ras GAP to down-regulate Ras, analogous to the PKC effect discussed above. This model is supported by recent evidence that in stimulated cells much of the ras GAP is associated with the p190 protein (discussed above) and that this complex is relatively ineffective at stimulating Ras GTPase activity (Polakis et al., 1990). Another exciting possibility is that association of ras GAP with p62 (discussed above) produces a complex that transduces the Ras response. In this model, phosphorylation of ras GAP on tyrosine results in complex formation, and interaction of this complex with p21^{ras} at the plasma membrane propagates a mitogenic signal. The excess unphosphorylated ras GAP in the cell would terminate the signal by competing for binding to the effector site of p21^{ras}. This model confounds the controversy as to whether ras GAP is an effector or inhibitor of Ras action by proposing that it is both (Zhang et al., 1990a). The weakness in this model is that ras GAP association with

p62 or p190 has not yet been reported in *v-ras*-transformed cells.

The characterization of *ras* GAP-like genes in *Saccharomyces cerevisiae* (*RA1* and *IRA2*) (reviewed in Wigler, 1990) along with the exciting discovery that the human neurofibromatosis gene is a *ras* GAP homolog (Xu et al., 1990) has provided evidence that some proteins in the GAP family are growth suppressors. Deletion of the yeast *IRA* genes causes a phenotype similar to that observed when transforming *ras* genes are introduced, arguing quite convincingly that *IRA* is an inhibitor rather than an effector of *ras* in *S. cerevisiae*. Likewise, since homozygous mutations/deletions in the neurofibromatosis gene result in enhanced growth of Schwann cells, it appears that this protein serves to suppress growth. Whether it does so by interacting with p21^{ras} remains to be seen. Interestingly, the homology between the neurofibromatosis gene product and *ras* GAP is confined to a smaller region of the total protein than is the homology between the neurofibromatosis gene product and yeast *IRA* proteins. Significantly, neither the neurofibromatosis gene product (that fraction which has thus far been sequenced) nor *IRA* has a consensus SH-2 domain. If the SH-2 domain confers the associations necessary for signaling, as suggested in the model above, then one would not expect these proteins to be effectors.

The ability of *krev-1* (also called *rap-1A*) to reverse *v-ras* transformation has further complicated the question of the role of *ras* GAP. The *krev-1* gene product is quite homologous to p21^{ras} and binds more tightly to *ras* GAP than does p21^{ras}. But *ras* GAP does not stimulate the GTPase activity of *Krev-1*: a distinct GAP does this (Quilliam et al., 1990). One could argue that p21^{krev-1} reverses transformation by competing with p21^{ras} for its effector and keeping the effector in an inactive state. This would support the model of a GAP complex being the effector. Alternatively, p21^{krev-1} and p21^{ras} could compete for binding to another target yet to be identified. Only a small cluster of amino acids near the previously identified effector domain account for the opposing effects of p21^{krev-1} and p21^{ras} in transformation (Zhang et al., 1990b). A point mutation in *Ras* not far from this cluster (Asn-17) stabilizes the GDP-bound state and dominantly suppresses *c-ras* transformation by an unknown mechanism (Feig and Cooper, 1988). Thus far the only good evidence that *ras* GAP has an effect other than turning off *Ras* is the ability of exogenously added *ras* GAP to block a muscarinic receptor-regulated potassium channel (Yatani et al., 1990).

Attempts to understand the mechanism of transformation by *ras* have been hampered by the failure to ascertain the function of the *Ras* effector. In *S. cerevisiae* a protein called cyclase-associated protein (CAP) has been implicated in modulating the *Ras* activation of adenylate cyclase (Wigler, 1990). However, *Ras* does not activate adenylate cyclase in mammalian cells. A number of physiological responses to *Ras* activation have been observed, including membrane ruffling (Bar-Sagi and Feramisco, 1985), increased levels of diacylglycerol (Fleischman et al., 1986), induction of *c-fos* mRNA (Stacey et al., 1987), and in-

creased activity of phospholipase A₂ (Bar-Sagi and Feramisco, 1985; Bar-Sagi et al., 1988), S6 kinase (Barrett et al., 1990), and p34^{cdc2} serine kinase (Barrett et al., 1990). The increase in diacylglycerol appears to result from activation of phosphatidylcholine breakdown (Lacal et al., 1987b; Lopez-Barahona et al., 1990). Some but not all the *Ras* effects appear to involve activation of PK (Fukumoto et al., 1990; Gauthier-Rouvière et al., 1990; Lacal et al., 1987a; Wolfman and Macara, 1987). Thus, PK may act both upstream (Downward et al., 1990) and downstream of *Ras*. Without knowing the function of the *Ras* effector, it is not possible to determine how distal these physiological responses are to the primary actions of *Ras*.

A possible clue to the function of *Ras* in mammalian cells may come from the study of other low molecular weight GTP-binding proteins that are structurally related to *Ras* (for review see Hall, 1990). Many of these proteins have been implicated in the formation, movement and fusion of intracellular vesicles. The *rho* gene product appears to be involved in actin filament rearrangement (Chardin et al., 1989; Paterson et al., 1990). These findings suggest that proteins in the *Ras* family may also play a general role in controlling movement of proteins within the cell.

A completely novel model for the function of *Ras* is suggested by these studies. The p21^{ras} protein, through interaction with its effector, could control assembly of a class of cell surface receptors at specific sites in the plasma membrane. The mechanism by which this could occur is unknown, but it would likely involve actin filament rearrangement. Thus, in nontransformed cells, growth factor binding to receptor would activate p21^{c-ras}, which would then trigger actin filament rearrangement resulting in receptor patching and eventually internalization. However, in *v-ras*-transformed cells, receptor patching would be triggered in the absence of growth factor because of the constitutive activity of the p21^{v-ras} protein. Since tyrosine kinase-type receptors appear to be activated by oligomerization and cross-phosphorylation (see above), the patching of these receptors would result in growth factor-independent activation. In this model, *v-Ras* would have a very general effect on the cell by making whatever receptors are already present on the cell surface spontaneously active. Consistent with this model, in B lymphocytes p21^{c-ras} copatches at the plasma membrane with surface immunoglobulins, and the patching is blocked by metabolic inhibitors and the inhibitor of actin polymerization, cytochalasin D (Graziadei et al., 1990). Also consistent with the model is the finding of increased tyrosine phosphorylation of proteins in *ras*-transformed cells, analogous to that found with persistent activation by growth factors (Cuadrado, 1990). The requirement that *Ras* be located at the membrane (via isoprenylation) in order to transform is consistent with this model. One interesting prediction of this model is that *c-Ras* acts downstream of growth factor receptors while *v-Ras* acts upstream. Thus far there is very little evidence for this model; however, it could explain the dramatic and pleiotropic cellular responses to *Ras*.

G Protein Oncogenes

Although many protein-tyrosine kinase are thought to propagate mitogenic and transforming signals by pathways that do not involve the classic G proteins, there is now evidence that, in some tissues, activating mutations of G proteins can cause cell transformation. The first evidence of this was the discovery of mutations in the α subunit of G_s in a pituitary tumor (Landis et al., 1989). The mutations were in a region of the protein involved in GTP hydrolysis. By inhibiting GTP hydrolysis, the protein apparently remains associated with adenylate cyclase for a longer period of time and thereby increases cAMP levels. Although cAMP is growth inhibitory in many cells, it has a mitogenic effect in growth hormone-secreting pituitary tissue. A systematic search of various human tumors for other mutations in the α chain of G proteins that would inhibit GTPase activity revealed an interesting group of mutants (Lyons et al., 1990). Most tumors investigated did not have mutations in either α_s or α_{12} . However, 18 out of 42 pituitary adenomas had mutations in the GTPase-inhibiting domain of α_s . In addition, three out of ten ovarian sex cord stromal tumors and three out of eleven adrenal cortical tumors had mutations in α_{12} . Mutations that decrease the GTPase activity of α_{12} are likely to decrease rather than increase cAMP levels. Also, α_{12} transduces other signals independent of the decrease in cAMP. The fact that the G_s and G_{12} mutations in human tumors are very restricted and nonoverlapping argues that these G proteins do not play a general role in transducing mitogenic signals but rather play specific roles in these tissues.

Conclusion

Despite the major advances that have been made in the past few years in understanding oncogene signaling, no clear picture has yet emerged for the biochemical mechanism of transformation. The implication of this review is that mammalian cell growth and transformation are controlled by a very complex set of events with a certain amount of redundancy. The recent discovery that some of the critical genes for regulation of the cell cycle have been functionally conserved from yeast to man raises hope that the basic features of cell growth control in mammalian cells will also be conserved throughout the eukaryotic kingdom. If so, then it should be possible to sort out the pathways that are central to cell growth control in all systems from those that are peripheral or tissue specific. Given the number of new signaling pathways that have been discovered in the past decade, it would be naive to assume that all the critical second messengers for growth control have already been discovered.

Perhaps the most useful new concept to emerge is that protein-tyrosine phosphorylation can in some instances create specific binding sites for proteins containing SH-2 domains and thereby regulate protein associations within the cell (Figures 2-4). Intramolecular interactions between phosphotyrosine and SH-2 domains may also regulate enzymatic activities (Figure 2). Since these interactions apparently require only short stretches of amino

acids, it should be possible to predict which tyrosine-phosphorylated protein will associate with which SH-2-containing protein. These sequences might also be useful targets for drug intervention. Conceivably, if peptide analogs capable of interacting with specific SH-2 domains could be introduced into cells, they would provide inhibition of specific cellular responses. This is likely to be an area of active research over the next few years.

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Genistein, Daidzein, and Their β -Glycoside Conjugates: Antitumor Isoflavones in Soybean Foods from American and Asian Diets[†]

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A method is described for the separation and analysis of isoflavone β -glycoside conjugates and aglucones in various foods derived from soybeans. After initial extraction using 80% aqueous methanol and defatting of the extract with hexane, the isoflavones were analyzed by gradient elution reversed-phase high-pressure liquid chromatography. Their structures were confirmed by fast atom bombardment ionization mass spectrometry and by proton nuclear magnetic resonance spectroscopy. The results reveal that most Asian and American soy products, with the exception of soy sauce, alcohol-extracted soy protein concentrate, and soy protein isolate, have total isoflavone concentrations similar to those in the intact soybean. Asian fermented soy foods contain predominantly isoflavone aglucones, whereas in nonfermented soy foods of both American and Asian origin isoflavones are present mainly as β -glycoside conjugates. Since the much larger estimated daily intake of these isoflavones by Asians compared to Americans is similar on a body weight basis to the isoflavones in soybean-containing diets which inhibit mammary tumorigenesis in animal models of breast cancer, it is possible that dietary isoflavones are an important factor accounting for the lower incidence and mortality from breast cancer in Asian women.

INTRODUCTION

The concept of reducing cancer risk by chemoprevention has become an important aspect of current cancer research (Boone *et al.*, 1990). The anti-estrogen, tamoxifen, introduced to therapeutically prevent the metastatic growth of breast cancer, is being studied as a possible chemopreventive agent for breast cancer (Powles *et al.*, 1989; Love, 1991). Compounds in the diet that have properties similar to, or are antagonists of, the physiologic estrogens may also have a role in reducing cancer risk. Two of these so-called phytoestrogens, lignans and isoflavones, have been suggested to play a role in the prevention of estrogen-dependent breast cancer (Setchell *et al.*, 1984; Barnes *et al.*, 1990; Adlercreutz *et al.*, 1991) and of colon cancer (Setchell *et al.*, 1981). This hypothesis has been supported by our subsequent data showing that soy, specifically containing isoflavones, inhibits tumor numbers in classical animal models of breast cancer (Barnes *et al.*, 1990, 1993). Furthermore, we have recently shown that two isoflavones, genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-dihydroxyisoflavone), inhibit the growth of human breast cancer (Peterson and Barnes, 1991) and prostate cancer (Peterson and Barnes, 1993) cell lines in culture, but by mechanisms independent of inhibition of the binding of steroids to their receptors.

Although flavonoids are found in plants, vegetables, and flowers in a bewildering display of biosynthetic prowess, isoflavones such as genistein and daidzein are found in just a few botanical families. This is because of the limited distribution of the enzyme chalcone isomerase [which converts 2(*R*)-naringenin, a flavone precursor, into 2-hydroxydaidzein] largely to tropical legumes. As a result, isoflavones are a very minor part of the modern American (M. Messina, unpublished data) or British diets (Jones *et al.*, 1989). Genistein and daidzein (Figure 1) and their β -glucoside conjugates are present in high concentrations (up to 3 mg/g) in soybeans (Walz, 1931; Walter, 1941; Eldridge, 1982; Price and Fenwick, 1985). Recent studies by Kudou *et al.* (1991) have shown that the 6''-O-malonylglucosides are the principal conjugated forms of these isoflavones in soybean hypocotyl. In addition, in toasted defatted soy flakes, Farmakalidis and Murphy (1985) have reported the presence of 6''-O-acetylglucosides.

In the Asian countries, soy is used in many foods—in Taiwan, the average human consumption is 35 g/day per capita (M. Messina, unpublished data). Lee *et al.* (1991) have recently shown a strong correlation between the intake of soy protein and a reduction in the risk of breast cancer in premenopausal, but not postmenopausal, Singapore Chinese women. Furthermore, soy ingestion has also been shown to significantly alter hormonal characteristics of healthy, premenopausal women in a manner which is beneficial with regard to risk factors for breast cancer (Cassidy *et al.*, 1993). Finally, the amount of isoflavones in urine is correlated to soy intake in both European (Setchell *et al.*, 1984; Axelson *et al.*, 1984) and Japanese people (Adlercreutz *et al.*, 1991). Therefore, soybeans are a potentially important link between diet and cancer risk.

Asians consume soybeans in many forms, including soy milk, tofu, and fermented products, such as miso, soy sauce, and tempeh. There have been only a limited number of reports of the isoflavone concentrations and composition

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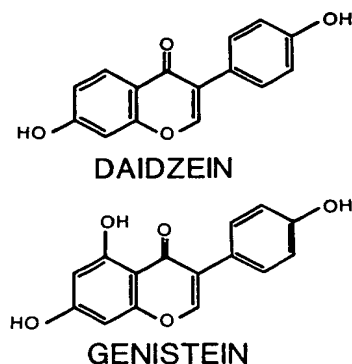


Figure 1. Chemical structures of the isoflavones measured in this study.

in these products. Since the consumption of soy-based foods is increasing in the United States, we describe here an analytical procedure for the determination of the concentrations and composition of isoflavones in a variety of American and Asian soy-containing foods and food products.

EXPERIMENTAL PROCEDURES

Apparatus. In this study the following were used: a Buchi rotavapor, Model R110; a LKB Ultraspec UV-visible wavelength spectrophotometer; a Perkin-Elmer Series IV high-pressure liquid chromatograph with a ternary solvent delivery system, UV-visible wavelength detector, and data station; a Varian MAT 311A mass spectrometer retrofitted with an Ion Tech saddle-field atom gun providing a beam of fast atoms of xenon (8 keV with a 1-mA current); and a GE 300 wide-bore spectrometer (NT series) with an 1180e processor and a 293c pulse programmer providing a resonance frequency of 300.1 MHz.

Reagents. HPLC grade methanol and acetonitrile and the disodium salt of fluorescein were used without further purification. Double-distilled water used was filtered through a nylon membrane of 0.45 μ m.

Soybean chips, various soy flours, soy protein concentrates, soy protein isolates, high-fiber fractions from soybeans, and soy molasses (a concentrated aqueous ethanol extract of defatted soybean flour) were provided by the Archer Daniels Midland Co. (Decatur, IL). Soy molasses contained 45% solids by weight (34.6% carbohydrate, 3.2% protein, 3.1% fat, and 4.2% ash), as analyzed by Hazelton Laboratories, Madison, WI. Tofu prepared by the δ -gluconolactone method (Morinaga Nutritional Foods Inc., Los Angeles, CA) and fermented soy sauce (Kikkoman) were obtained from a local grocery store. Tofu prepared by calcium salt-induced coagulation (Tree of Life, St. Augustine, FL), tempeh, soy milk, chocolate malted soy milk, iced soy milk (Tofutti and Ice Bean), soy cheese, miso, soy flour, soy nuts, and soy powder were all obtained from a national foodstore specializing in vegetable-based food products. Barley miso and rice miso were provided by American Soy Products, Inc., Saline, MI. Miso and several forms of soybean paste (*Doen Jang*) were purchased from a Korean foodstore.

Isoflavone Standards. *Isolation.* Genistin and daidzin, the β -glucoside conjugates of the isoflavones genistein and daidzein, were isolated from soy molasses according to the method of Walter (1941). Genistein was prepared by hydrolysis of genistin under reflux for 6 h in methanolic HCl. Daidzein was prepared by hydrolysis of daidzin with β -glucosidase in a 0.1 M sodium acetate buffer, pH 5.0. The crude daidzein and genistein were recrystallized three or four times from hot 60% aqueous ethanol.

Mass Spectrometry. Molecular weights of the isoflavones were determined by fast atom bombardment ionization mass spectrometry using a glycerol matrix.

^1H Nuclear Magnetic Resonance (NMR) Spectra. Isoflavones were dissolved in DMSO- d_6 . ^1H NMR spectra were obtained with 8K data points, 2800-Hz sweep width, 3.7-s repetition rate, and 75° sweep angle; spectra were internally referenced to tetramethylsilane at 0.00 ppm.

Extraction. Isoflavones in solid foods (analyzed in triplicate) to which 1.25 mg of fluorescein was added as an internal standard were extracted into 80% aqueous methanol (10 mL/g) by stirring 1 h at 60 °C. The other soy products (miso, soy milk, soy paste and tofu) were extracted whole and also after freeze-drying. The mixture was centrifuged (10 min at 2500g) and the supernatant decanted into a round-bottom flask. The pellet was resuspended in 80% aqueous methanol (2 \times 5 mL) and centrifuged, and the supernatants were combined and taken to dryness using a rotary evaporator. The dried extracts were then redissolved in 50% aqueous methanol (5 mL), and lipids were removed and discarded by partitioning into hexane (4 \times 20 mL). The aqueous methanol phase was evaporated to dryness on a rotary evaporator and the dried residue dispersed in 10 mL of 80% aqueous methanol. An aliquot of the mixture was centrifuged at 14000g for 2 min in an Eppendorf microfuge just prior to analysis by HPLC.

High-Pressure Liquid Chromatography. Separation of isoflavones was achieved by HPLC on a 30 cm \times 0.45 cm Brownlee Aquapore C₈ reversed-phase column with a mobile phase consisting of a gradient of 0–46.4% acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid at a flow rate of 1.5 mL/min. The concentration of acetonitrile increased by 2.25%/min. The eluting components were detected from their absorbance at 262 nm. Concentrations of the isoflavones were calculated from standard curves of the area responses for authentic isoflavone standards normalized to the constant amount of fluorescein added to each sample. The concentrations were expressed either as milligrams per gram of whole food or as milligrams per gram of dry weight.

RESULTS

Purity of Isolated Isoflavone from Soybeans. *Melting Points.* The uncorrected melting points of the isoflavones were as follows: daidzein, 325 °C (dec) [literature (Yueh and Chu, 1977) 320 °C (dec)]; daidzin, 223 °C (dec); genistein, 297–299 °C [literature (Walz, 1931) 296–298 °C]; genistin, 257–258 °C [literature (Walter, 1941) 254–256 °C].

UV Absorbance. Maximum absorbance occurred at 254 nm for daidzin, genistin, and genistein (molar extinction coefficients of 29.0×10^3 , 41.7×10^3 , and 37.3×10^3 , respectively, in 80% aqueous methanol), and at 250 nm for daidzein (molar extinction coefficient of 26.0×10^3 in 80% aqueous methanol).

Mass Spectrometry. Molecular masses of the isolated isoflavones were 270 and 432 daltons (genistein and genistin, respectively) and 254 and 416 daltons (daidzein and daidzin, respectively).

NMR Spectroscopy. Genistein: δ 6.22 (1H, d, J = 2.4 Hz, H₆), 6.38 (1H, d, J = 1.5 Hz, H₈), 6.82 (2H, d, J = 8.4 Hz, H_{3'} and H_{5'}), 7.37 (2H, d, J = 8.4 Hz, H_{2'} and H_{6'}), 8.30 (1H, s, H₂), 9.57 (1H, s, C₄-OH), 10.86 (1H, s, C₇-OH), and 12.93 (1H, s, C₅-OH). Daidzein: δ 6.81 (2H, d, J = 9.3 Hz, H_{3'} and H_{5'}), 6.87 (1H, s, H₈), 6.94 (1H, d, J = 10.5 Hz, H₆), 7.38 (2H, d, J = 8.4 Hz, H_{2'} and H_{6'}), 7.97 (1H, d, J = 8.7 Hz, H₅), 8.27 (1H, s, H₂), 9.59 (1H, s, 4'-OH), and 10.86 (1H, s, 7-OH). These values are consistent with previously published data (Markham and Mabry, 1975). By using DMSO- d_6 as the solvent, the phenolic hydroxyl ^1H resonances were observed, whereas addition of D₂O eliminated these resonances. As expected, the ^1H NMR spectra of genistin and daidzin lacked the C₇ phenolic hydroxyl proton resonance, the site of attachment of the glucosyl group. Small chemical shift changes (δ 0.1–0.4) caused by the 7-glucosyl group were also observed for protons (H₅, H₆, and H₈) in the A ring.

Optimization of Extraction and Analytical Procedures. *p*-Nitrophenol, estradiol, and fluorescein were evaluated as possible candidates for use as internal standards. Fluorescein was found to be the most suitable

Table I. Isoflavone Concentrations^a in Asian Primary Soy Materials

food	basis	conjugated		aglucones		total	D/G ratio	aglucones, %	
		genistin	daidzin	genistein	daidzein			genistein	daidzein
soy milk	g	0.130 ± 0.004	0.103 ± 0.006	0.007 ± 0.000	0.011 ± 0.002	0.252 ± 0.012	0.83	5	10
	g dry wt	1.680 ± 0.060	1.337 ± 0.087	0.098 ± 0.002	0.141 ± 0.019	3.256 ± 0.168			
tofu ^b	g	0.249 ± 0.028	0.121 ± 0.010	0.031 ± 0.001	0.016 ± 0.001	0.417 ± 0.036	0.49	11	12
	g dry wt	1.215 ± 0.137	0.591 ± 0.046	0.151 ± 0.006	0.077 ± 0.005	2.031 ± 0.171			
tofu ^c	g	0.269 ± 0.004	0.200 ± 0.008	0.015 ± 0.001	0.015 ± 0.000	0.494 ± 0.011	0.74	5	7
	g dry wt	2.087 ± 0.030	1.513 ± 0.019	0.116 ± 0.004	0.113 ± 0.000	3.827 ± 0.045			
soy flour	g	0.741 ± 0.100	0.582 ± 0.077	0.015 ± 0.002	nd	1.338 ± 0.178	0.77	2	0
soy powder	g	1.148 ± 0.103	0.582 ± 0.054	0.014 ± 0.001	nd	1.748 ± 0.156	0.50	1	0
soy nuts	g	1.390 ± 0.039	0.853 ± 0.022	0.066 ± 0.001	0.054 ± 0.001	2.363 ± 0.061	0.62	5	6

^a mg/g; mean ± 1 SD of triplicate analyses. ^b Tree of Life tofu. ^c Mori-Nu tofu.Table II. Isoflavone Concentrations^a in Processed or Fermented Asian Soy Products

soy product	basis	conjugated		aglucones		total	D/G ratio	aglucones, %	
		genistin	daidzin	genistein	daidzein			genistein	daidzein
tempeh	g	0.113 ± 0.028	0.040 ± 0.013	0.164 ± 0.004	0.113 ± 0.007	0.430 ± 0.005	0.55	59	74
	g dry wt	0.296 ± 0.063	0.103 ± 0.029	0.434 ± 0.005	0.298 ± 0.009	1.130 ± 0.096			
miso	g	0.043 ± 0.004	0.035 ± 0.025	0.497 ± 0.029	0.345 ± 0.013	0.920 ± 0.070	0.70	92	91
	g dry wt	0.064 ± 0.007	0.054 ± 0.038	0.745 ± 0.068	0.516 ± 0.036	1.379 ± 0.149			
rice miso	g	0.198 ± 0.011	0.000 ± 0.000	0.136 ± 0.000	0.071 ± 0.002	0.404 ± 0.009	0.21	41	100
	g dry wt	0.353 ± 0.018	0.000 ± 0.000	0.242 ± 0.001	0.127 ± 0.003	0.721 ± 0.014			
barley miso	g	0.155 ± 0.020	0.142 ± 0.025	0.239 ± 0.008	0.185 ± 0.007	0.721 ± 0.053	0.83	61	57
	g dry wt	0.258 ± 0.032	0.235 ± 0.042	0.396 ± 0.012	0.306 ± 0.009	1.195 ± 0.084			
Shiromiso soup mix	g	0.267 ± 0.020	0.163 ± 0.028	0.170 ± 0.006	0.108 ± 0.008	0.708 ± 0.059	0.62	39	40
Akamiso soup mix	g	0.319 ± 0.025	0.254 ± 0.044	0.173 ± 0.005	0.136 ± 0.008	0.882 ± 0.080	0.79	35	35
soybean paste	g	0.078 ± 0.014	0.044 ± 0.040	0.251 ± 0.008	0.197 ± 0.009	0.570 ± 0.071	0.73	76	82
	g dry wt	0.160 ± 0.030	0.090 ± 0.081	0.514 ± 0.016	0.404 ± 0.019	1.168 ± 0.146			
soybean paste/rice	g	0.066 ± 0.029	0.085 ± 0.016	0.108 ± 0.004	0.103 ± 0.006	0.362 ± 0.041	1.08	62	55
	g dry wt	0.106 ± 0.045	0.136 ± 0.026	0.174 ± 0.008	0.166 ± 0.008	0.582 ± 0.061			
soybean paste/wheat	g	0.110 ± 0.008	0.094 ± 0.026	0.124 ± 0.014	0.105 ± 0.001	0.433 ± 0.032	0.85	53	53
	g dry wt	0.220 ± 0.015	0.189 ± 0.052	0.248 ± 0.028	0.210 ± 0.003	0.867 ± 0.063			

^a Expressed as mg/g wet weight or mg/g dry weight; mean ± 1 SD of triplicate analyses. nd, not detected.Table III. Isoflavone Concentrations^a in Other Soy Foods

soy food	basis	conjugated		aglucones		total	D/G ratio	aglucones, %	
		genistin	daidzin	genistein	daidzein			genistein	daidzein
soy sauce	g	nd	nd	0.009 ± 0.002	0.014 ± 0.001	0.023 ± 0.003	1.50	100	100
	g dry wt	nd	nd	0.036 ± 0.014	0.054 ± 0.013	0.090 ± 0.026			
soy cheese	g	0.028 ± 0.001	0.021 ± 0.001	0.002 ± 0.001	0.001 ± 0.001	0.050 ± 0.003	0.71	8	2
	g dry wt	0.057 ± 0.001	0.043 ± 0.001	0.005 ± 0.001	0.001 ± 0.002	0.105 ± 0.003			
Tofutti	g	0.022 ± 0.001	0.004 ± 0.006	0.004 ± 0.000	0.001 ± 0.002	0.032 ± 0.008	0.19	18	20
	g dry wt	0.064 ± 0.001	0.012 ± 0.016	0.014 ± 0.001	0.003 ± 0.004	0.092 ± 0.020			
Ice Bean	g	0.060 ± 0.006	0.055 ± 0.007	0.001 ± 0.000	0.001 ± 0.002	0.117 ± 0.014	0.91	2	2
	g dry wt	0.184 ± 0.016	0.167 ± 0.022	0.004 ± 0.002	0.004 ± 0.006	0.360 ± 0.004			

^a Expressed as mg/g wet weight or mg/g dry weight; mean ± 1 SD of triplicate analyses. nd, not detected.

because its HPLC retention index was distinct from that of any other component in the soy food extracts.

When alcohol-extracted soy protein concentrate (1 g) was "spiked" with known amounts of genistein, genistin, daidzein, and daidzin, dried, and then extracted with several different solvent mixtures (10 mL), the maximum isoflavone recoveries were found for 80% aqueous methanol and 60% aqueous acetonitrile. Recoveries ranged from 90 to 93% and from 86 to 90%, respectively, for these two solvent systems.

Experiments were carried out to determine the optimum ratio of the volume of the extraction solvent (80% aqueous methanol) to the quantity of food. A range from 0.2 to 1 mg of each isoflavone was present in the soy flour used in this experiment. Consistently higher recoveries were obtained with ratios of solvent (milliliters) to food (grams) equal to or greater than 10:1. Recoveries were lower at ratios below 10 mL of solvent/g of food; however, it was possible to correct for losses using the internal standard, fluorescein. The coefficient of variation for triplicate samples varied from 3.0 to 8.6%, being lower when larger sample sizes (>0.5 g) were analyzed. Similar variation

was observed for the values for foods reported in Tables I-IV, being highest (12.0%) in nonhomogeneous foods containing low isoflavone concentrations (>0.4 mg/g) and lowest (5.6%) in powdered materials with isoflavone concentrations greater than 1 mg/g.

Asian Soy Foods. Each of the Asian-style soybean products (soy milk, tofu, soy flour, soy powder, and soy nuts), which were not diluted by the addition of nonsoybean components, had total concentrations of isoflavone (expressed as milligrams per gram of dry weight) in the range 1.3-3.8 mg/g (Table I). When other food components were added to the soybean product, the overall isoflavone concentrations were lower. Fermented soy foods, which are usually prepared by mixing soy with other components such as barley, rice, or wheat, contained isoflavone concentrations that were lower and in the range 0.6-1.4 mg/g of dry weight (Table II). Other soy-based products, such as frozen flavored soy milk (Ice Bean and Tofutti), soy sauce, and soy cheese, had much lower isoflavone concentrations (Table III).

For soy milk (Figure 2A), soy flour, soy nuts, soy powder, and tofu (Figure 2B), the β -glucosidic conjugates were the

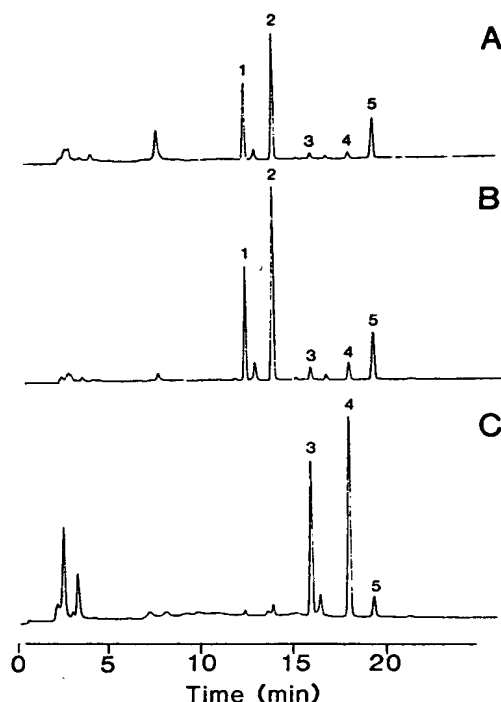


Figure 2. Reversed-phase HPLC chromatograms of extracts of soy milk (A), tofu (B), and miso (C). Note that miso contains only unconjugated isoflavones. Peak identification: 1, daidzin; 2, genistin; 3, daidzein; 4, genistein; 5, internal standard (fluorescein; constant amount added to each sample). Each chromatogram was obtained at the same sensitivity setting; the volumes injected of each extract were adjusted to give similar maximum peak heights.

major forms of isoflavones present. However, in fermented soy foods such as miso (Figure 2C), soybean paste, and tempeh, the unconjugated isoflavone aglucones were the predominant chemical forms.

American Soy Foods. The commercial soy products used in American foods all contained isoflavones (Table IV). Soy flours (Figure 3A), independent of the degree of heating used in their preparation, had consistently high isoflavone concentrations. Soy protein concentrate prepared by extraction with water had an isoflavone concentration (2.7 mg/g) comparable to that of soy flour and many Asian soy products. On the other hand, aqueous alcohol-extracted soy protein concentrate (Figure 3B) contained lower concentrations of isoflavones (Table IV). American soy sauce had the lowest concentrations of isoflavones (Figure 3C). In soy protein isolates, isoflavone concentrations were reduced 3-fold compared with soy flour when the concentration was expressed as milligrams per gram of dry weight and 5-fold when expressed as milligrams per gram of protein (Table IV).

DISCUSSION

By application of the method described and evaluated here, we have comprehensively examined the isoflavone composition of a variety of soybean products typically consumed in American and Asian diets. The data extend results reported by other investigators (Murphy, 1982; Eldridge, 1982; Eldridge and Kwolek, 1983; Farmakalidis and Murphy, 1985; Price and Fenwick, 1985; Setchell *et al.*, 1987; Jones *et al.*, 1989; Wang *et al.*, 1990).

The procedure developed in this study for the analysis of isoflavones in food products is reproducible and reliable and is suited to the measurement of isoflavones in as little as 1 g or 1 mL of the foodstuffs studied. The optimum

conditions for the extraction and isolation of the isoflavone from food products were thoroughly investigated using soy protein concentrate prepared by extraction with 65% aqueous alcohol. However, we cannot exclude possibility that alternative solvent mixtures may be optimal for other soy food matrices. The optimum ratio of the aqueous organic solvent to the food being analyzed was 10:1 (vol/g) or greater. As previously noted (Wang *et al.*, 1990), the use of Sep-Pak C₁₈ columns to clean up extracts was unnecessary (data not shown). Although defatting of the initial extract with hexane did not alter the qualitative or quantitative aspects of the HPLC analysis, it is recommended to prolong the life of the HPLC column.

Efficient extraction of these relatively polar isoflavones from foodstuffs requires the use of a polar solvent, in accord with previous studies (Murphy, 1981; Eldridge, 1982; Eldridge and Kwolek, 1983; Setchell *et al.*, 1989; Jones *et al.*, 1989; Barbuch *et al.*, 1989), aqueous methanol (80%) was shown to be the optimum solvent for the extraction of conjugated and unconjugated isoflavones, whereas pure acetonitrile, ethanol, or methanol was a poor organic solvent. Although it has been previously claimed that 80% aqueous acetonitrile was more efficient than 80% aqueous methanol, absolute recoveries were not reported (Murphy, 1981; Farmakalidis and Murphy, 1985). In addition, previously published methods utilized acidified extraction medium (Murphy, 1981; Farmakalidis and Murphy, 1985; Wang *et al.*, 1990). In whole soybeans (and presumably some, but not necessarily all, soy food products) large amounts of isoflavone 6''-O-malonylglucoside conjugates have been reported (Kudou *et al.*, 1991). The malonate ester derivatives, by analogy to malonate esters which are intermediates in many organic syntheses, are prone to decarboxylation to form the corresponding 6''-O-acetylglucosides, a reaction stimulated by heat and acid medium. This may account for 6''-O-acetyldaidzin and 6''-O-acetylgenistin in toasted soy flakes (Farmakalidis and Murphy, 1985), as was suggested by Kudou *et al.* (1991). Therefore, the apparent pattern of isoflavone glycosidic conjugates measured by HPLC analysis will be a function of the extraction procedure, as well as the processing of individual soy foods. Recent experiments carried out in this laboratory (S. Barnes, unpublished data) suggest that hot extraction procedures also cause decarboxylation of 6''-O-malonylglucosides and 6''-O-acetylglucosides, thereby leading to the underivatized β -glucosides (daidzin and genistin) as the predominant forms detected by HPLC analysis, as observed in this and previous studies (Walz, 1931; Walter, 1941; Eldridge, 1982; Price and Fenwick, 1985; Setchell *et al.*, 1987; Jones *et al.*, 1989). Despite these effects, the total isoflavone glycoside concentration was unchanged. Accordingly, the concentrations of daidzin and genistin as determined by HPLC in this study represent the sum of the individual isoflavone glycoside concentrations. New extraction and analytical methods are required that will enable accurate determination of the composition and concentration of isoflavone glycosidic conjugates in soy food materials.

Solvent loss by retention in the insoluble food matrix or by evaporation, was corrected for by the inclusion of fluorescein as the internal standard. Under the chromatographic conditions employed, fluorescein eluted separately from other UV-absorbing compounds extracted from the foods tested and did not partition into hexane, and its bright yellow-green color served to limit potential problems of losses during the workup procedure. With the exception of Eldridge (1982), who used butyrophenone as an internal

soy product	conjugated		aglucones		total		D/G ratio	aglucones, %	
	genistin	daidzin	genistein	daidzein	dry wt	protein		genistein	daidzein
soybean chips	0.356 ± 0.074	0.331 ± 0.058	0.052 ± 0.019	0.065 ± 0.023	0.802 ± 0.172	2.111 ± 0.455	0.97	13	16
soy flours									
Nutrisoy	1.448 ± 0.026	1.161 ± 0.003	0.034 ± 0.000	0.033 ± 0.002	2.678 ± 0.027	5.356 ± 0.054	0.58	2	3
Nutrisoy 7B	1.318 ± 0.009	1.112 ± 0.005	0.053 ± 0.002	0.044 ± 0.001	2.527 ± 0.004	5.054 ± 0.008	0.60	4	4
baker's Nutrisoy	1.300 ± 0.176	1.046 ± 0.117	0.024 ± 0.020	0.019 ± 0.019	2.389 ± 0.332	4.778 ± 0.664	0.56	2	2
toasted Nutrisoy	1.385 ± 0.066	1.093 ± 0.025	0.044 ± 0.011	0.040 ± 0.005	2.561 ± 0.076	5.122 ± 0.152	0.57	3	3
soy concentrates									
water extracted	1.404 ± 0.103	1.180 ± 0.082	0.033 ± 0.002	0.039 ± 0.002	2.656 ± 0.182	3.794 ± 0.256	0.61	2	3
alcohol extracted									
Arcon F	0.087 ± 0.014	0.064 ± 0.007	0.004 ± 0.001	0.004 ± 0.000	0.159 ± 0.022	0.244 ± 0.034	0.75	4	6
Arcon S	0.227 ± 0.059	0.102 ± 0.019	0.069 ± 0.001	0.045 ± 0.002	0.443 ± 0.075	0.682 ± 0.115	0.50	23	31
soy isolate	0.430 ± 0.138	0.232 ± 0.105	0.105 ± 0.011	0.073 ± 0.004	0.848 ± 0.228	0.931 ± 0.250	0.41	20	24
soy isolate	0.589 ± 0.004	0.278 ± 0.001	0.189 ± 0.012	0.102 ± 0.005	1.158 ± 0.012	1.273 ± 0.013	0.35	24	27
soy fiber	0.154 ± 0.004	0.141 ± 0.001	0.114 ± 0.006	0.085 ± 0.002	0.494 ± 0.012		0.84	43	38

Figure 3 consists of three vertically stacked HPLC chromatograms labeled A, B, and C. The x-axis for all three is 'Time (min)' ranging from 0 to 20. Chromatogram A (soy flour) shows five distinct peaks labeled 1 through 5. Peak 2 is the tallest, followed by peak 1. Peaks 3, 4, and 5 are much smaller. Chromatogram B (soy protein concentrate) shows four peaks labeled 1, 2, 3, and 5. Peak 5 is the tallest, followed by peak 2. Peaks 1 and 3 are smaller. Chromatogram C (soy sauce) shows five peaks labeled 1 through 5. Peak 5 is the tallest. Peaks 1, 2, 3, and 4 are relatively small and appear as a cluster between 10 and 15 minutes. The baseline in C is more noisy than in A and B.

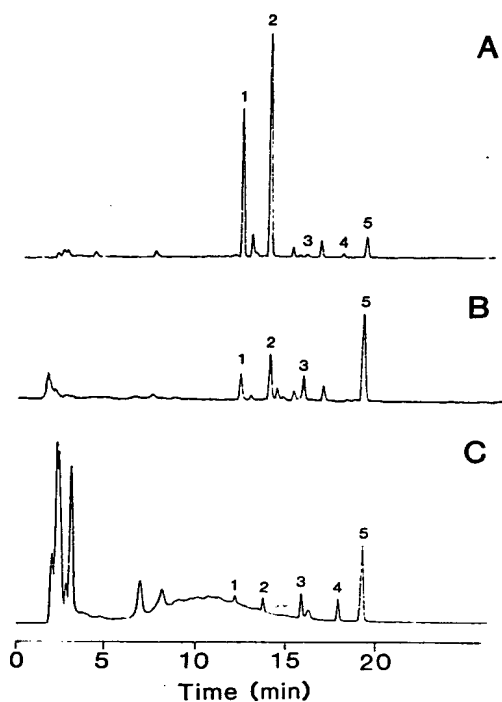


Figure 3. Reversed-phase HPLC chromatograms of extracts of soy flour (A), aqueous alcohol-extracted soy protein concentrate (B), and soy sauce (C). The same sensitivity setting was used for each chromatogram. The amount of fluorescein added to each sample was constant; i.e., the isoflavone content of soy flour vastly exceeds that of the protein concentrate and soy sauce. Peak identification is as in Figure 2.

standard, previously published methods for isoflavones have taken no account of procedural losses. The isoflavones were separated by reversed-phase HPLC using a gradient of acetonitrile and water, thus permitting analysis of both the β -glucosides and the aglucones in a single chromatographic run. This has particular advantage over methods in which the isoflavone glucosides were calculated by the difference in the concentration of flavone aglucones, determined using isocratic HPLC methods, between acid-hydrolyzed and unhydrolyzed samples (Wang *et al.*, 1990). It is an important feature of the analytical method because of the marked differences in isoflavone composition in soy foods. Other investigators have previously used gradient elution reversed-phase HPLC. Murphy (1981) and Eldridge and Kwolek (1983) employed gradients of methanol in water, whereas Koster *et al.* (1983), Jones *et al.* (1989), Matsuura *et al.* (1989),

and Kudou *et al.* (1991) all utilized acetonitrile gradients in water. The analyses were carried out at different pHs. Jones *et al.* (1989) used a borate-potassium phosphate buffer, pH 7.5, whereas the other investigators used either trifluoroacetic acid [the present study and Matsuura *et al.* (1989)] or acetic acid (Koster *et al.*, 1983; Kudou *et al.*, 1991).

Application of our analytical method has indicated that manufacture of most American and Asian soy food materials does not result in substantial lowering of isoflavone concentrations. The exceptions were soy sauce, alcohol-extracted soy protein concentrate, soy protein isolates, soy fiber, and foods in which soy was found to be only a minor component.

Soy flour is obtained by grinding dehulled soybeans following the removal of oil by solvent extraction or by extrusion. Isoflavones were not found in soybean oil. During the manufacturing process, soy flour is heated to varying degrees, including toasting, to produce different grades of soy flours. Toasting of soy flour may well have led to formation of more isoflavone 6''-O-acetylglucosides by decarboxylation of the 6''-O-malonylglucosides than in less heat-treated forms of soy flour. Nonetheless, these glycosidic conjugates were de-esterified according to the extraction procedure employed in the present study; accordingly, we found no evidence that heating had an effect on the isoflavone composition (glucosidic conjugates vs aglucones) or total concentrations determined by our analytical method.

Soy protein concentrate, prepared by extraction of soy flour with hot 65% aqueous ethanol (an excellent solvent for isoflavones), had 10–20-fold lower isoflavone concentrations than most other soy foods. By contrast, soy protein concentrate prepared by hot water extraction (to remove the soluble carbohydrates) at neutral pH retained most of the isoflavones present in soy flour, probably reflecting their strong protein binding and low aqueous solubility.

Soy protein isolate is prepared by solubilization of proteins (and soluble carbohydrates) from soy flour by an alkaline (pH 9.5) extraction step and the subsequent acid (pH 4.5) precipitation of the extracted proteins. The isoflavone concentrations in soy protein isolate were 4–6-fold lower than in soy flour or soy protein concentrate when expressed as milligrams per gram of protein. Incomplete recovery of the isoflavones from the soy flour during the alkaline extraction step or selective precipitation of the isoflavone aglucones at pH 4.5 may account for the composition and concentrations of isoflavones in the soy protein isolate. The lower daidzein/genistein ratio

(0.4) in the soy protein isolate compared to that in the unprocessed soy flour (0.6) may also be a consequence of the more hydrophilic nature (and hence aqueous solubility) of daidzein compared to genistein.

Some soy foods (soy milk, miso) containing high concentrations of isoflavones are often diluted with other food materials that do not contain isoflavones. The addition of chocolate flavoring to soy milk lowers the isoflavone concentration (milligrams per milliliter) by 33%; however, since the total solids were increased, there was a 3-fold decrease in isoflavone concentration when expressed as milligrams per gram of freeze-dried weight. Tofutti and Ice Bean are both iced soy milks. Tofutti is prepared using soy protein isolate rather than the whole soybean (as is the case for Ice Bean) and consequently has a 4-fold lower isoflavone concentration than Ice Bean. The lower isoflavone concentrations reported in infant formula soy milk (Setchell *et al.*, 1987) also reflect the use of soy protein isolate rather than whole soybeans, which were the source for the soy milks analyzed in the present study.

When miso was diluted with barley or rice, the isoflavone concentrations (1.2 and 0.7 mg/g of dry weight, respectively) were also lower than in undiluted miso (1.4 mg/g of dry weight). Similar effects were also found for soybean paste (diluted with rice or wheat), which had isoflavone concentrations ranging from 0.58 to 1.17 mg/g of dry weight.

Soy sauce, a popular product in Asia and in the United States, is prepared by first fermenting soybean paste with rice and then squeezing the resulting cake to produce a brown liquor. It contains only trace amounts of isoflavones, as has been noted previously (Wang *et al.*, 1990). Murphy (1982) failed to detect isoflavones in soy sauce.

In nonfermented soy foods (soy milk, tofu, soy nuts, soy powder, soy protein concentrate, and soy flour), the isoflavones were present almost exclusively as their glycosidic conjugates, whereas in fermented soy products, such as miso, soybean paste, and tempeh, a large proportion of the isoflavones were in the unconjugated form. Interestingly, when soy milk or tofu becomes contaminated by microorganisms after the container is opened, the proportion of unconjugated isoflavones increases with time (L. Coward and S. Barnes, unpublished data). This may have been the case for the tofu analyzed by Murphy (1982) that contained a high percentage of isoflavone aglucones.

Asians consume the equivalent of 10–35 g of soybeans/day per capita (M. Messina, unpublished data), indicating a daily isoflavone intake of 25–100 mg. This is comparable (on a body weight basis) to the amounts of isoflavones in powdered soybean chip-containing diets that we have recently shown to inhibit the appearance of mammary tumors in a *N*-methylnitrosourea-induced animal model of breast cancer in rats (Barnes *et al.*, 1990).

In contrast to most Asians, the average American or Western European consumer ingests at most only a few milligrams per day of isoflavones. This has been confirmed by several investigators. Setchell *et al.* (1984) showed that the urinary excretion of the isoflavone metabolite equol increased >500-fold when human volunteers included 40 g of soy flour in their diet on a daily basis. In addition, urinary levels of equol are generally very low in most subjects consuming a Western-style diet but are much higher in vegetarians who include soy in their diet (Adlercreutz *et al.*, 1987). Finally, Jones *et al.* (1989) have shown that the isoflavone intake from the British diet is below that believed to be necessary to have a physiological effect.

Since we have shown that isoflavone-containing soybean products prevent the appearance of mammary tumors in rat models of breast cancer (Barnes *et al.*, 1990) and isoflavones inhibit the growth of human breast cancer (Peterson and Barnes, 1991) and prostate cancer (Peterson and Barnes, 1993) cells in culture, these data are consistent with the hypothesis that the low incidence of breast cancer in Asian women is due to their much higher consumption of soybeans containing isoflavones (Setchell *et al.*, 1984; Barnes *et al.*, 1990; Adlercreutz *et al.*, 1991; Lee *et al.*, 1991).

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テンペイの抗酸化性物質の生成に關与する β -グルコシダーゼ

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β -Glucosidase Involved in the Antioxidant Formation in Tempeh, Fermented Soybeans

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The β -glucosidase involved in formation of antioxidants (some isoflavones) from fermented soybeans with *Rhizopus oligosporus* was investigated.

The results were as follows:

1. Genistin was hydrolysed with the crude enzyme which was obtained by ammonium sulfate fractionation from water extract of *Rhizopus oligosporus* cultivated in the medium containing soybean paste. The reaction mixture showed antihemolytic activity corresponding to the proposed amount of its aglucone, 5,7,4'-trihydroxyisoflavone.
2. The β -glucosidase was isolated by the purification procedures of acetone precipitation, Amberlite CG-50 column chromatography and concentration with collodion bag. The enzyme was purified about 1400 folds and the yield was 43%.
3. The purified enzyme showed maximum activity at pH 5.0 and 60°C, and was stable between pH 2 and 9 at 20°C. The enzyme activity was markedly inhibited by heavy metal ions and SH reagents, and recovered by cysteine. The K_m values of the enzyme were $6.25 \times 10^{-5} M$ for genistin, $1.98 \times 10^{-4} M$ for NPG, $4.57 \times 10^{-4} M$ for phenyl- β -D-glucoside and $6.72 \times 10^{-4} M$ for arbutin. Methyl- α -D-glucoside was not attacked by the enzyme.

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緒 言

テンペイ (tempeh) は, インドネシアにおいて常用されている発酵大豆食品であり, これは大豆を加熱脱皮し, *Rhizopus* 菌を接種して, 30~37°C, 24~48 時間発酵させて作ったものである。このテンペイの発酵過程において, 未発酵大豆にはみられない抗溶血作用* の増大が認められ⁽¹⁾, また抗溶血因子としてテンペイから三つ

の結晶が分離され, このうち二つは, それぞれ脱脂大豆からすでに分離されたことのあるゲニステイン (genistein), ならびにダイゼイン (daidzein) と同定したが, 他の一つは未知のまま Factor 2 と命名し, 後にこれまで天然体から分離されたことのない 6,7,4'-trihydroxyisoflavone と一致することを明らかにした⁽²⁾。

テンペイのアルコール溶出物による抗溶血作用は, 未発酵大豆のアルコール溶出物では, ほとんど検出困難であること, また大豆からゲニステインやダイゼインの配糖体であるゲニスチン (genistin) やダイジン (daidzin) が, かなり多量抽出分離されている^(3,4) 事実から, 大豆に *Rhizopus* 菌を植えて発酵させる過程で, おそらくこの菌の生産する β -グルコシダーゼの作用によって, 種

* ビタミン E 欠ラットの血球は, dialuric acid によって溶血を受けるが, ビタミン E その他の抗酸化性物質の添加 (in vitro) によって, その溶血は防止される。そして, in vitro における抗溶血活性と抗酸化性は, ほぼ相関することも認められている。

々のイソフラボンの配糖体から, それらのアグリコンであるゲニステイン, ダイゼインならびに 6-7,4'-trihydroxyisoflavone などが遊離して, これらが抗溶血性ならびに抗酸化性を示すものと推定した. そこで, まずゲニステインその他, 入手容易な β -グルコシドの水解酵素活性を目標として, *Rhizopus oligosporus* の菌体の水抽出液から β -グルコシダーゼの精製をおこなった. その結果, 用いた基質のうちゲニステインに対し, もっとも親和力の高い酵素標品を得, その性質について調べたので報告する.

実験方法および結果

1) β -グルコシダーゼの測定方法

(1) *p*-ニトロフェニール β -D-グルコシド (NPG) を基質とした場合: 精製過程における活性の測定には, NPG を 0.1 M 酢酸緩衝液 (pH 5.0) に 8.3×10^{-4} M になるように溶解させ, その 0.5 ml に同じ酢酸緩衝液で希釈した酵素液 0.5 ml を加え, 40°C, 20 分反応後, 0.2 M 炭酸緩衝液 (pH 10.2) 2.0 ml を加えて反応を停止させ, 遊離した *p*-ニトロフェノールの発色を 400 m μ で測定した. この条件下において, 吸光値 1.0 に相当する *p*-ニトロフェノールを遊離するに要する酵素量をもって, 1 単位 (U) とした. 酵素たんぱく質は Lowry らの銅-Folin 改良法⁽⁵⁾ により, カゼインたんぱく質を標準として測定した. この方法で求めたたんぱく質 1 mg 当りの活性 (U) をもって比活性 (U/mg) とし, 菌体水抽出粗酵素液の比活性と, 各精製過程における酵素液の比活性の比をもって純化度とし, 菌体水抽出粗酵素の総活性に対する精製酵素の総活性の百分率をもって収率とした.

(2) ゲニステインを基質とした場合: ゲニステインは水に溶けにくく, エチルアルコールに溶解するが, エチルアルコールが 10% 以上になると酵素反応に阻害が生じるので, 酵素反応に際し, ゲニステインの 80% エタノール溶液 0.5 ml に 0.1 M 酢酸緩衝液 (pH 5.0) 1.0 ml を加え, これに酵素液の適量を加えて水で全量を 4.0 ml とし, 40°C で反応させた. 酵素反応により, ゲニステインから遊離したグルコースを測定したが, 未反応のゲニステインが Somogyi のアルカリ銅液で呈色するため, ホモバニリン酸による蛍光法⁽⁶⁾を用いた. すなわち, 反応液の 1 ml を採取し, これをあらかじめ加熱浴中につけておいた 0.1 M トリス緩衝液 (pH 8.5) 1.8 ml を含む試

験管に入れて反応を停止させ, 2.5 mg/ml ホモバニリン酸 0.1 ml, 0.75 mg/ml パーオキシダーゼ 0.1 ml を順次加え, 1 mg/ml グルコースオキシダーゼ 0.1 ml を加え, 30°C, 60 分反応後, 分光蛍光光度計 (日立 203 型) を用いて λ_{ex} ; 315 m μ , λ_{em} ; 425 m μ の蛍光を 5 μ g/ml, 硫酸キニーネの蛍光を 100% として測定した. 別に, 標準グルコースより求めた蛍光検量曲線にこの値を挿入して, 生成グルコース量を定量した.

(3) その他の基質の場合: アルブチン, フェニール- β -D-グルコシド, メチル- α -D-グルコシドを基質とした場合は, これらの基質を 0.1 M 酢酸緩衝液 (pH 5.0) に溶解させ, この 0.5 ml に酵素液 0.5 ml を加えて 40°C, 一定時反応させたのち, Somogyi のアルカリ性銅液 2.0 ml を加えて反応を停止させ, グルコースを標準として, 比色法により還元糖の定量⁽⁷⁾を行なった.

2) *Rhizopus oligosporus* の培養

Rhizopus oligosporus NRRL 2710 菌を用い, 培地としては馬鈴薯-ブドウ糖培地を用いた. すなわち, 馬鈴薯 300 g を約 500 ml の純水でよく煮て, ガーゼで濾過し, 濾液を水で 1000 ml とし, これにブドウ糖 20 g を添加して作成した培地に対し, その培地重量の 1/3 重量の加熱磨砕大豆を加えて混合し, 各ルーフラスコに 200 g ずつの割合で分注した (加熱磨砕大豆は, テンペイ作成の場合に準じて大豆 300 g を 0.25% 酢酸液 100 ml 中で 1 時間加熱後, 大豆の種皮を除き, これに 0.25% 酢酸液 100 ml を加えて, さらに 1 時間加熱したのち, ミキサーでよく磨砕して作った). このようにして調製滅菌した培地に *Rhizopus* 菌の 2 白金鈎を接種し, 37°C, 65 時間静置培養した.

3) 水抽出粗酵素液の調製

上述のごとく培養した菌体を集めて, 圧搾して搾汁を集め, 一方圧搾湿菌体をミキサーで磨砕し, その 1/3 重量の水を加えて酵素を抽出し, 両者をあわせて菌体水抽出粗酵素液とした.

4) ゲニステインに対する β -グルコシダーゼの作用ならびに生成ゲニステイン量と溶血防止力の関係

Rhizopus oligosporus の β -グルコシダーゼの精製に先立ち, ゲニステインに粗酵素を作用せしめた反応生成物の溶血防止力について検討を行なった. 80% エタノールにとかした 0.06% ゲニステイン溶液 0.5 ml に, 0.1 M 酢酸緩衝液 (pH 5.0) 1.5 ml を加え, 水抽出粗酵素液より 0.3~0.7 飽和硫酸塩析, 透析した酵素溶液 2.0 ml を

作用させ 37°C, 20 時間反応のち熱失活させ, ゲニステイン以外の物質を除去するため, 反応液に同容の 99% エタノールを加えてよく混合し, エーテルで 2, 3 回抽出し, エーテル層を集めて水で洗浄し, エーテルを留去した残渣を一定量の 99% エタノールで抽出し, 262 m μ における吸光値からゲニステイン量を求めた. 溶血テストは Rose ら⁽⁸⁾ の方法に従った. すなわち, ビタミン E 欠食で飼育されたネズミの赤血球の生理的食塩水懸濁液は, dialuric acid によって破壊され溶血をおこすが, ビタミン E その他の抗酸化剤によって溶血が防止されるので, この程度を水による完全溶血の 540 m μ による E 値などと比較することにより溶血率を求めた. ゲニステ

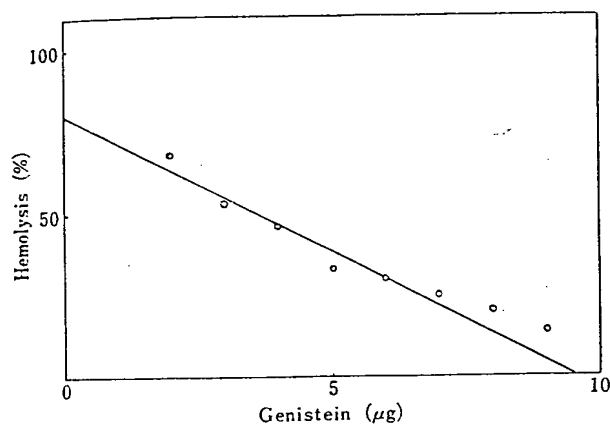


Fig. 1. Effect of Enzymic Hydrolysate of Genistein on Hemolysis.

Two ml of crude enzyme, 0.5 ml of 0.06% genistin in 80% ethanol and 1.5 ml of 0.1M acetate buffer (pH 5.0) were mixed and incubated at 37°C for 20 hr.

The reaction was stopped in boiling water. To the reaction mixture, the same volume of 99% ethanol was added and the reaction product, genistein was extracted with ether. The sample was prepared by evaporating ether from the extracts.

The hemolysis test of samples was carried out according to the procedure of Rose and György.⁽⁸⁾

ンから硫安分画粗酵素で遊離したゲニステインの 2~9 μ g 相当量の各レベルを, ビタミン E 欠赤血球に接触後, dialuric acid を作用させたときの溶血率の関係を Fig. 1 に示す. これにより, 硫安 30~70% 分画酵素中の β -グルコシダーゼにより, ゲニステインが加水分解されて遊離したゲニステインの 6 μ g 相当量以上で, ビタミン E 欠ラットの赤血球懸濁液の溶血率を 30% 以下に防止し得ることが認められた.

5) β -グルコシダーゼの精製

(1) アセトン分別沈殿: 菌体水抽出粗酵素液に 4M 酢酸緩衝液 (pH 4.0) を終末 0.1M になるように加え, 6N HCl で pH 4.0 にしたのち, 0~4°C で冷アセトンを 20% になるように加え, 30 分間静置したのち遠心分離 (8000 rpm, 15 分) し, その上澄を 10N NaOH で pH 5.0 にしてから, 再び冷アセトンを加えて 60% にし, 30 分静置後前と同様遠心分離した. かくして得られた沈殿を脱イオン水に懸濁し透析し, 不溶沈殿を遠心により除去した. その結果は Table I に示すごとく, 比活性は約 20 倍に上昇し, 収率はほぼ 100% であった.

(2) Amberlite CG-50 カラムクロマトグラフィー: 常法により洗浄した Amberlite CG-50 をカラムに充填し, 0.004M 酢酸緩衝液 (pH 4.0) で十分緩衝化したのち, その上部に上にのべたアセトン沈殿画分の透析内液 (pH 5.3) を入れ, 0.5 ml/min の流速で pH 5.0 の酢酸緩衝液の濃度を 0.1M から 2M まで段階的に上げることにより, β -グルコシダーゼの溶出を行なった. その結果は Fig. 2, Table I に示したとおりで, 1M の緩衝液で溶出される酵素の比活性は, 約 314 倍に上昇した.

(3) コロジオンバッグによる濃縮, 透析: イオン交換樹脂によって溶出された活性画分のピーク (P-3-1) を集めて, さらにコロジオンバッグを用いて減圧濃縮することにより, 比活性 1410 の β -グルコシダーゼを収率

Table I. Purification of β -Glucosidase

Purification step	Volume (ml)	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Purification (times)
Water extract	1,757	3,710	22,800	0.162	100	1
20~60% Acetone precipitate	281	4,260	1,280	3.33	115	20.4
Effluent from Amberlite CG-50						
Peak 3	1,397	2,430	122	19.9	65.5	123
Peak 3-1	238	1,872	36.6	51.2	50.5	314
Concentrated and dialyzed solution		1,597	6.97	229	43.1	1410

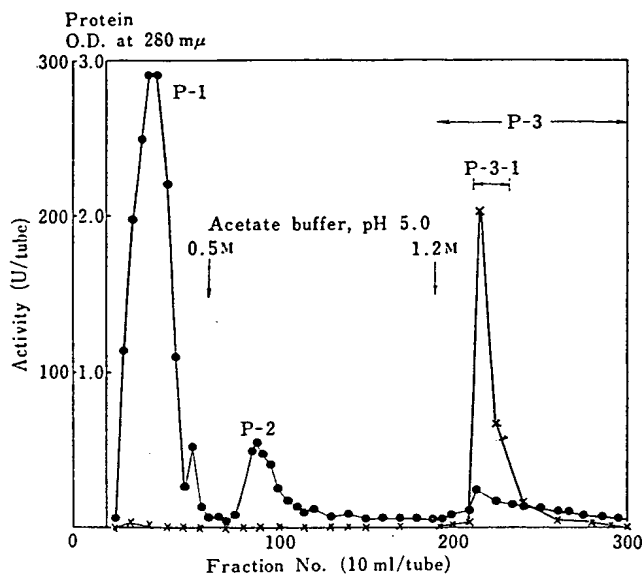


Fig. 2. Elution Patterns of β -Glucosidase from Amberlite CG-50 Column.

Acetone (20~60%) precipitate was loaded on column (30 \times 285 mm) equilibrated with 0.004 M acetate buffer at pH 4.0.

Stepwise-elution was employed with 0.5 M and 1.2 M acetate buffer of pH 5.0.

●—● protein, ×—× activity.

43% で得ることができた。かくして得られた標品について、0.1 M 酢酸緩衝液 (pH 5.0), 54,000 rpm で超遠心分析を行なった結果、沈降定数 $s_{20,w} = 3.5 \times 10^{-13}$ を得、ほぼ単一たんぱく質であることが認められた。

6) 精製酵素の2, 3の性質

(1) pH 安定性: 各 pH の緩衝液 (0.1 M) 中に同容の酵素を加えて、20°C, 20 時間保ったのち、NPG を基質として残存活性を測定した結果は、Fig. 3 に示すように、安定 pH 域は 2~9 に認められた。

(2) 最適 pH と温度: 本酵素活性の最適 pH と温度を NPG を基質として測定した結果を、Fig. 4 と Fig. 5 に示した。最適 pH は 5.0~5.2 にあり、また最適温度は 60°C 付近にあって、それ以上になると酵素の熱失活による活性の低下がいちじるしく、70°C ではほとんど活性は失われる。

(3) NPG に対する β -グルコシダーゼの作用: NPG 溶液 (終濃度 1.66×10^{-4} M) に酵素 0.51 U を作用させて、経時的にその分解率をしらべた結果、分解率 20% 程度の範囲内では、反応速度は時間、酵素濃度にそれぞれ比例的関係のあることが認められた。さらに、温度変化に対する反応速度の関係を Arrhenius の式より

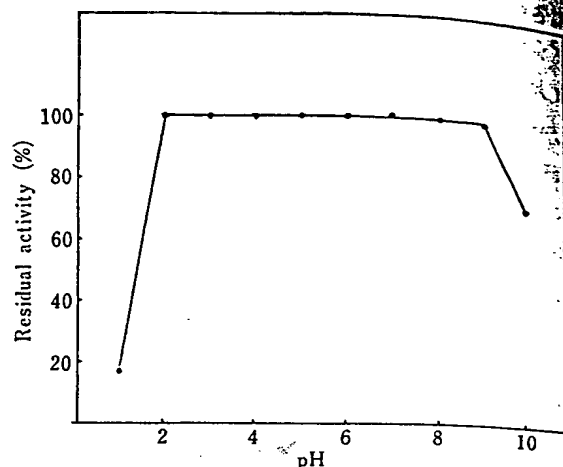


Fig. 3. Effect of pH on Stability of β -Glucosidase.

Potassium-chloride-HCl buffer for pH 1.0~2.0, citrate-phosphate buffer for pH 3.0~7.0, boric acid-borax buffer for pH 8.0, and carbonate-bicarbonate buffer for pH 9.0~10.0 were used.

Enzyme solution containing 0.12 U was mixed with the same volume of each buffer and the mixtures were kept at 20°C for 20 hr. After standing, 0.1 ml of the mixture, 0.5 ml of 8.3×10^{-4} M NPG and 0.4 ml of 0.1 M acetate buffer (pH 5.0) were mixed and incubated at 40°C for 20 min. Residual activity is represented as per cent of the activity of untreated enzyme solution.

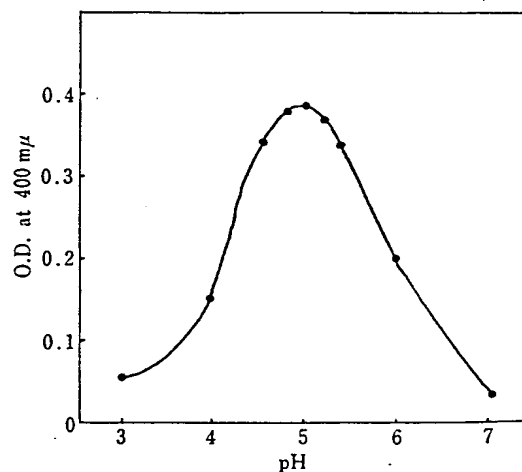


Fig. 4. Effect of pH on Activity of β -Glucosidase.

Citrate phosphate buffer was used for pH 3~7.

The reaction mixture was composed of 0.2 ml of 3.3×10^{-4} M NPG, 1.3 ml of each buffer and 0.5 ml of enzyme soln. and kept at 45°C, for 10 min.

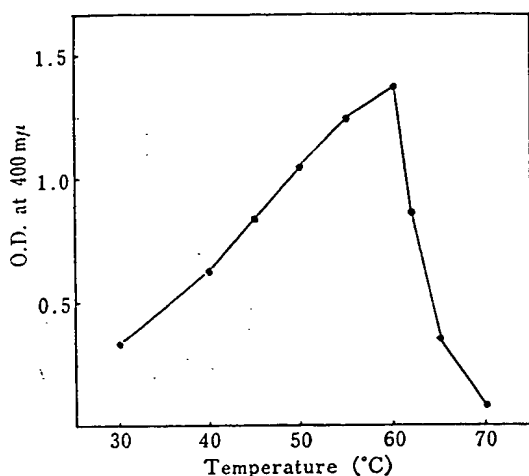


Fig. 5. Effect of Temperature on Activity of β -Glucosidase.

NPG, 0.5 ml of 8.3×10^{-4} M (pH 5.0), 0.5 ml of enzyme solution were mixed and kept for 20 min at each temperature.

求めた結果, 活性化エネルギーは 11.7 kcal/mole であった。

(4) 各種阻害剤の影響: 10^{-3} M の金属イオンの共存下において, NPG に対する β -グルコシダーゼの分解作用をしらべた結果, Table II に示すように, Ag^+ , Hg^{2+} , Cu^{2+} などに顕著な阻害作用が認められた。また, SH 基に特異的な阻害剤についてしらべるために, 酵素液と阻害剤を 40°C , 10 分プレインキュベーションしたのち, 基質 NPG を加えて反応させ阻害実験とし, さらに賦活化の実験として NPG とシステインを添加して反応を行なった。結果は Table III に示すごとく, PCMB などにより阻害された反応が, システイン添加によって明らかに賦活されており, SH 基が活性に関係し

Table II. Inhibition of the Purified Enzyme by Various Cations

Cation	Inhibition (%)	Cation	Inhibition (%)
None	0	Cu^{2+}	42.1
Li^+	2.4	Co^{2+}	10.0
Na^+	0.4	Ni^{2+}	2.0
K^+	2.9	Fe^{3+}	5.8
Ba^{2+}	15.1	Ag^+	100
Ca^{2+}	4.2	Hg^{2+}	98.4
Mg^{2+}	5.6	Mn^{2+}	14.9
Zn^{2+}	10.5		

The reaction mixture (1.0 ml) contained 0.51 units of enzyme, 1×10^{-3} M cation as indicated and 4.15×10^{-4} M NPG in 0.1 M acetate buffer (pH 5.0).

Table III. Effects of Sulfhydryl Agents on the Purified Enzyme

Inhibitor	Concentration ($\times 10^{-3}$ M)	Cysteine (mg)	Inhibition (%)
None		0	0
Arsenite	1	0	5.8
	1	1	2.3
ICH_2COOH	1	0	8.5
	1	1	4.1
PCMB	2	0	48.5
	2	1	2.6

After 0.51 units of enzyme and inhibitor were preincubated for 10 min, NPG (4.15×10^{-4} M) solution with or without cysteine was added to the preincubated enzyme mixture (pH 5.0) and reacted for 20 min at 40°C .

Table IV. Inhibition of the Purified Enzyme at Various Concentration of Sugars

Sugars	Concentration (M)	Inhibition (%)
None		0
D-Glucose	2.8×10^{-5}	0
	1.4×10^{-4}	7.2
	2.8×10^{-4}	9.5
	4.15×10^{-4}	12.5
	7.0×10^{-4}	16.9
	1.4×10^{-3}	27.0
Xylose	4.15×10^{-4}	5.6

Reaction mixture (1.0 ml) contained 0.75 units of enzyme, sugars as indicated and 4.15×10^{-4} M NPG in 0.1 M acetate buffer (pH 5.0).

ているものと考えられた。一方, 本酵素反応の生成物であるグルコースならびにキシロースについても, 阻害の影響をしらべた結果, Table IV に示すように, グルコース濃度の増加とともに β -グルコシダーゼ作用は阻害され, キシロースにもグルコースの 1/2 程度の阻害が認められた。

(5) β -グルコシダーゼの各種 β -グルコシドに対する K_m : NPG, フェニル β -グルコシド, アルブチンとフラボノイド β -グルコシドとして, ゲニスチンを基質として, Lineweaver-Burk の式を用いて, ミハエリス定数 K_m を求めた結果を, Table V に一括した。これより本酵素は, 種々の基質の中でもゲニスチン, すなわち Factor 2 のグルコシドに最も構造類似の基質に対する親和性の大きいことが認められた。また, 基質濃度がある程度以上になると反応速度が低下し, Lineweaver-Burk のプロットが V 字形を示すことが観察された。また 7×10^{-3} M のメチル- α -D-グルコシドに本酵素を作用させたが, グルコースの遊離は全く認められなかった。

Table V. Affinities of Various Glucosides for the Purified Enzyme

	K_m (M)
Genistin	6.25×10^{-5}
NPG	1.98×10^{-4}
Phenyl- β -D-glucoside	4.57×10^{-4}
Arbutin	6.72×10^{-4}

Genistin was used at concentration of $2.5 \sim 6.25 \times 10^{-5}$ M; NPG of $0.2 \sim 2.0 \times 10^{-3}$ M; phenyl- β -D-glucoside of $0.1 \sim 2.0 \times 10^{-3}$ M; arbutin of $0.4 \sim 10 \times 10^{-3}$ M.

The reaction mixtures contained 1.25~4.40 units of enzyme, various glucoside as indicated and 0.1 M acetate buffer (pH 5.0), and incubated for 5~10 minutes at 40°C.

Enzyme activities of the mixtures were measured as described in "Experimental."

考 察

天然体に含まれる種々の抗酸化性の抗溶血性物質のうち、ビタミンEと同程度の活性を有するものとして、テンペイから抽出分離された 6,7,4'-trihydroxyisoflavone は、大豆に多く含まれるゲニステインのアグリコンであるゲニステイン、すなわち 5,7,4'-trihydroxyisoflavone の異性体である。6,7,4'-trihydroxyisoflavone の dimethoxy(6,4'-)誘導体 afomasin は、1960年 McMurry ら⁽⁹⁾により、African hardwood より分離された。6,7,4'-trihydroxyisoflavone は、afomasin 同定の過程で afomasin の水解により結晶状に得られているほかには、これまで天然体から直接分離された報告を見ない。本物質のテンペイからの収量は、ゲニステインの約1/100程度であるが、抗溶血活性はゲニステインの約20倍である。また大豆に含まれるダイゼインのアグリコンであるダイゼインも、抗溶血活性はゲニステインの1/5程度であるので、これらがテンペイ貯蔵中の抗酸化⁽¹⁾に果す役割は大きいものと考えられる。一方、未発酵大豆粉末は貯蔵の過程で容易に酸化を受け、過酸化価(PV)やTBA値(2,4-チオバルビツール酸法)の上昇が著しい⁽¹⁾。このことは、大豆に含まれるゲニステイン、ダイゼインその他のイソフラボンの配糖体に、*Rhizopus oligosporus* の生産する β -グルコシダーゼが作用して、アグリコンであるゲニステイン、ダイゼインならびに 6,7,4'-trihydroxyisoflavone などを遊離するためと推定し、研究を行なったものであるが、抗溶血活性の最も強い 6,7,4'-trihydroxyisoflavone の配糖体を未だ分離していな

いため、これを基質としたときの酵素活性や K_m についていないが、テンペイより分離した Factor 2、すなわち 6,7,4'-trihydroxyisoflavone は、その異性体であるゲニステイン同様、*Rhizopus* 菌の生産する β -グルコシダーゼによって、不活性の配糖体から遊離した 6,7,4'-trihydroxyisoflavone もしくは 5,7-dihydroxy 基やダイゼインにおける 7-hydroxy 基が酸化防止的に作用し、特に Factor 2 における 6,7-ortho-diphenolic group の diketone 構造は、過酸化物の free radical の破壊に役立つものと考えられる⁽¹⁰⁾。従って、*Rhizopus* 菌の生産する β -グルコシダーゼの果す役割は大きい。今後 Factor 2 の配糖体を分離したうえ、本酵素の基質特異性について、さらに検討したい。

要 約

(1) テンペイ製造に使用される *Rhizopus oligosporus* の菌体水抽出液を硫酸分画した粗酵素をゲニステインに作用させて、生成するゲニステインを溶血テストを用いて調べた結果、酵素反応液中のゲニステイン量と溶血防止力とはほぼ相関し、本酵素標品中にはゲニステインの水解に関与する β -グルコシダーゼの存在することが認められた。

(2) 本酵素の性質を明らかにするために、アセトン分別沈殿、Amberlite CG-50 カラムクロマトグラフィー、コロジオンバッグによる濃縮法により精製し、約1400倍に純化された酵素を43%の収率で得た。

(3) この精製酵素の諸性質を検討した結果、最適 pH は 5.0 であり、pH 安定性は pH 2~9 で比較的広範囲にわたっていた。至適温度は 60°C で、それ以上でいちじるしい活性の低下が認められた。また重金属や SH 試薬で阻害され、システインにより賦活されることから、SH 酵素であることが示唆された。

(4) 各基質について、ミハエリス定数 K_m を求めた結果、ゲニステインに対する $K_m = 6.25 \times 10^{-5}$ M、NPG に対する $K_m = 1.98 \times 10^{-4}$ M、フェニール β -D-グルコシドに対する $K_m = 4.57 \times 10^{-4}$ M、アルブチンに対する $K_m = 6.72 \times 10^{-4}$ M で、イソフラボン配糖体のゲニステインに対する親和力が、用いた他のいずれの基質に対するよりも、大であることを認めた。

(5) この酵素はメチル α -D-グルコシドに作用しないことから、 β -グルコシド結合を特異的に切断する β -グルコシダーゼであることを認めた。

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STRUCTURAL STUDY OF ISOFLAVONOIDS POSSESSING ANTIOXIDANT ACTIVITY ISOLATED FROM THE FERMENTATION BROTH OF *STREPTOMYCES* SP.

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Structures of three antioxidant isoflavonoids isolated from the cultured broth of *Streptomyces* sp. OH-1049 were shown to be 4',7,8-trihydroxyisoflavone (1), 3',4',7-trihydroxyisoflavone (2) and 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3), respectively. Among them, 3 is a novel isoflavonoid possessing a chlorine atom in the molecule.

Compound 1 was synthesized and its antitumor activities were tested against IMC carcinoma, S180, P388 leukemia and P388/ADM leukemia *in vivo*. As a result, 1 showed 139% increase in life span (ILS) against S180 bearing mice whereas it showed slight or no ILS against IMC carcinoma, P388 leukemia and P388/ADM leukemia bearing mice.

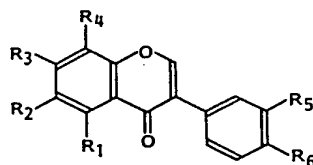
In the course of a screening program for novel antibiotics showing antioxidant activity, three active components were isolated from the fermentation broth of *Streptomyces* sp. OH-1049 and characterized as 4',7,8-trihydroxyisoflavone (1), 3',4',7-trihydroxyisoflavone (2) and 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3), respectively.

The taxonomy of the producing organism, fermentation, and isolation of the active components and antioxidant and anti HeLa S₃ activities of these antibiotics were reported in the preceding paper¹⁾. This paper deals with the physico-chemical properties and structure elucidation of 1~3 and synthesis and antitumor activity tests of 1.

Materials and Methods

General Experimental Procedures

MP's were determined using a Yanagimoto MP-3 hot stage microscope and are uncorrected. UV spectra were recorded on a Shimadzu model UV-200S spectrophotometer and IR spectra on a Jasco



- 1 $R_1=R_2=H$ $R_3=R_4=OH$ $R_5=H$ $R_6=OH$
- 2 $R_1=R_2=H$ $R_3=OH$ $R_4=H$ $R_5=R_6=OH$
- 3 $R_1=OH$ $R_2=H$ $R_3=OH$ $R_4=Cl$ $R_5=R_6=OH$
- 4 $R_1=R_2=H$ $R_3=OAc$ $R_4=H$ $R_5=R_6=OAc$
- 5 $R_1=OAc$ $R_2=H$ $R_3=OAc$ $R_4=Cl$ $R_5=R_6=OAc$
- 6 $R_1=OH$ $R_2=Cl$ $R_3=OH$ $R_4=R_5=H$ $R_6=OH$
- 7 $R_1=OH$ $R_2=Cl$ $R_3=OH$ $R_4=Cl$ $R_5=H$ $R_6=OH$

model A-102 interferometer. MS were obtained with a Jeol model DX-300 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian XL-400 instrument. DC-Fertigplatten Kieselgel 60 (Merck) was used for TLC analysis and for preparative TLC. TRI Rotar-V (Jasco) and Uvidec-100 (Jasco) instruments were used for HPLC with a column of YMC A-303 (Yamamura Chemical Laboratory; 4.6 i.d. \times 250 mm) eluted with MeOH - H_2O (39 : 11) as solvent.

Isolation of 1~3

Isolation procedures of 1~3 were described in the preceding paper¹⁾.

Preparation of 3',4',7-Triacetoxyisoflavone (4)

Compound 2 (10 mg) was acetylated using pyridine (0.5 ml) and Ac_2O (0.5 ml) to afford 3',4',7-triacetoxyisoflavone (4, yield 12.0 mg).

Preparation of 8-Chloro-3',4',5,7-tetraacetoxyisoflavone (5)

Compound 3 (18 mg) was acetylated using pyridine (0.5 ml) and Ac_2O (0.5 ml) to afford 8-chloro-3',4',5,7-tetraacetoxyisoflavone (5, yield 9.0 mg).

Preparation of 4',7,8-Trihydroxyisoflavone (1)

4',7,8-Trihydroxyisoflavone (1) used for antitumor activity tests was prepared by applying the ethyl orthoformate method reported by KARMARKAR²⁾.

Antitumor Activity Tests of 4',7,8-Trihydroxyisoflavone (1)

Female CDF₁ and ICR mice (6-week old) were purchased from Shizuoka Laboratory Animal Center.

Tumor cells were maintained in ascitic form by serial ip passaging in mice. Tumor cell lines and mice used in the present experiment are described in Table 2. In all tumor models, the agent was administered ip as 9 doses on days 1~9 after tumor inoculation.

Antitumor activity of the samples on ascitic tumor was evaluated by the increase in life span (ILS): $(\text{T/C}-1) \times 100\%$, where "T" is the mean survival days (MSD) of the treated group and "C" is the MSD of the control group.

Results

Physico-chemical Properties of 1~3

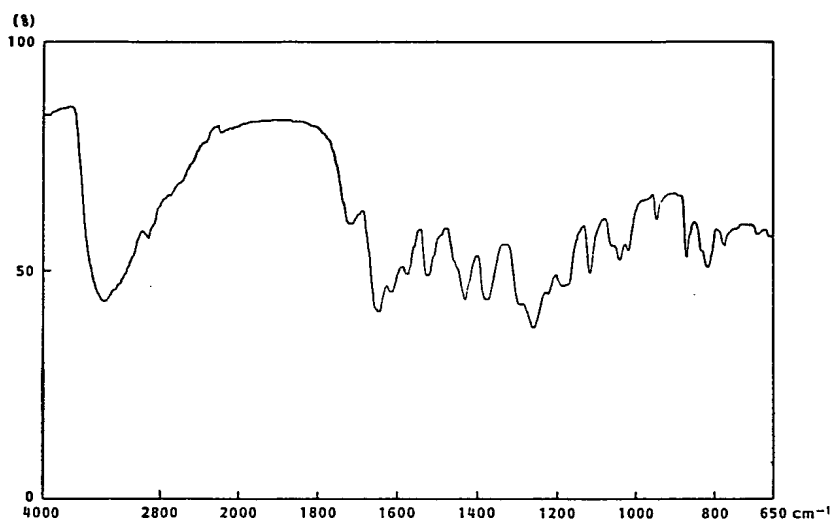
Physico-chemical properties of 1~3 are summarized in Table 1 and IR absorption spectrum of 3 is shown in Fig. 1. Compounds 1~3 gave positive color reaction with iodine, 50% sulfuric acid and FeCl_3 solution and was negative to ninhydrin reagent.

Table 1. Physico-chemical properties of 1~3.

	1	2	3
Appearance	Colorless powder	Colorless powder	Pale yellow powder
Molecular formula	$\text{C}_{15}\text{H}_{10}\text{O}_5$	$\text{C}_{15}\text{H}_{10}\text{O}_5$	$\text{C}_{15}\text{H}_9\text{O}_5\text{Cl}$
MW	270	270	320.5
Rf value ^a	0.23	0.24	0.36
UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm	260	248, 259, 292	264, 293 (sh)
$\lambda_{\text{max}}^{\text{MeOH-NaOH}}$ nm	276	257, 335	278, 331 (sh)
$\lambda_{\text{max}}^{\text{MeOH-HCl}}$ nm	258	248, 259, 292	264, 293 (sh)
IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1}	3460, 3180, 1674, 1578, 1560	3440, 3230, 1620, 1590	3340, 1645, 1612, 1259

^a CHCl_3 - MeOH (9 : 1).

Fig. 1. IR spectrum of 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3) (KBr).



Structure Elucidation of 1~3

Three active components 1~3 were proved to be isoflavonoids because of their characteristic UV absorption spectra³⁾ and the existence of lower field singlet in their ¹H NMR at δ 8.22, 8.07, and 8.17, respectively.

In the fast atom bombardment (FAB)-MS of 1, 293 ($(M+Na)^+$) was observed and the molecular formula of this compound was estimated to be $C_{15}H_{10}O_5$. On the other hand, in the ¹H NMR spectrum of this compound signals attributed to seven hydrogens were observed including a set of A_2B_2 type signals attributed to B-ring of the isoflavone skeleton (δ 6.93 (2H, d, $J=8$ Hz) and 7.46 (2H, d, $J=8$ Hz)) and a set of doublet (δ 6.93 (1H, d, $J=8$ Hz) and 7.53 (1H, d, $J=8$ Hz)). The bathochromic shift of the UV absorption maximum (Band II) from 260 to 270 nm by addition of NaOAc indicated the presence of 7-OH moiety in the structure³⁾ and the set of doublet signals was assigned to 6-H and 5-H, respectively. From the accumulated data described above, structure 1 was concluded to be 4',7,8-trihydroxyisoflavone.

The molecular formula of 2 was established to be $C_{15}H_{10}O_5$ through high resolution (HR)-MS analysis (M^+ obsd 270.052, calcd for $C_{15}H_{10}O_5$ 270.053). In the ¹H NMR spectrum of 2, three signals coupled each other at δ 6.81 (1H, dd, $J=2$ and 8 Hz), 6.82 (1H, d, $J=2$ Hz) and 8.03 (1H, d, $J=8$ Hz) were assigned to 6-H, 8-H and 5-H, respectively and the other set of three signals (δ 6.84 (1H, d, $J=8$ Hz), 6.92 (1H, dd, $J=2$ and 8 Hz) and 7.01 (1H, dd, $J=2$ Hz)) was assigned to the B-ring of the isoflavone skeleton. Finally, the structure of 2 was concluded to be 3',4',7-trihydroxyisoflavone through the NMR spectroscopic studies of the triacetyl derivative of 2 (4).

It was found that compound 3 contained a chlorine atom in the molecule through MS analysis and the molecular formula of this compound was established by HR-MS to be $C_{15}H_9O_5Cl$ (M^+ obsd 320.004 and 322.002, calcd for $C_{15}H_9O_5Cl$ 320.008 and 322.006). By acetylation of this compound, tetraacetate (5, M^+ 488 and 490) was obtained and in the ¹H NMR spectrum of 5, a singlet at δ 6.59 (1H, 6-H or 8-H), a set of three signals (δ 7.28 (1H, d, $J=8$ Hz, 5'-H), 7.41 (1H, dd, $J=1$ and 8 Hz, 6'-H) and 7.42 (1H, d, $J=1$ Hz, 2'-H)) attributed to the B-ring, and a typical lower field singlet at

Fig. 2. Fragments observed in the electron impact MS of 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3).

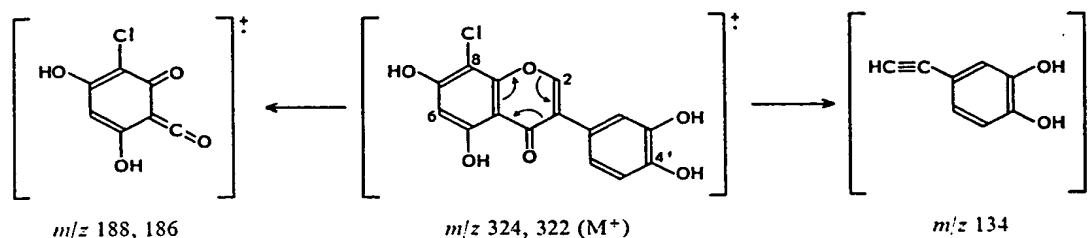
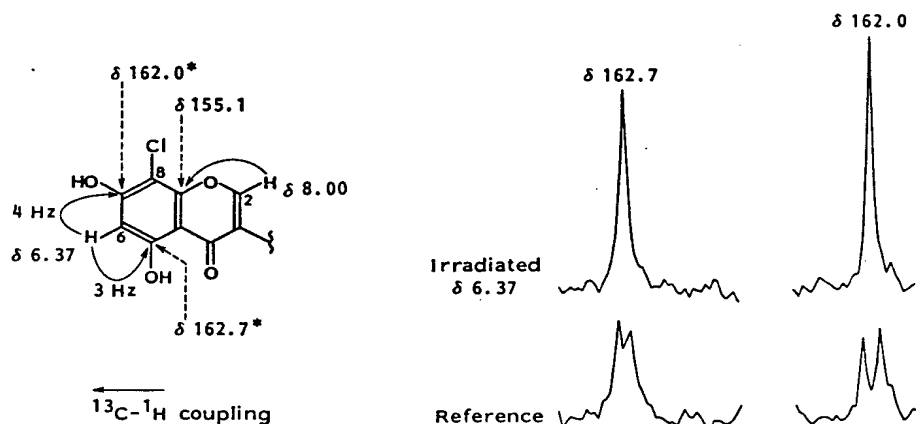


Fig. 3. LSPD experiments of 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3).



δ 8.00 (2-H) were observed. These observations indicated that a chlorine atom was attached to the A-ring. The MS fragments m/z 134, 186 and 188 derived from the cleavage of 3 (Fig. 2) also supported this hypothesis. In the UV absorption spectral study of 3, 12 nm bathochromic shift was observed when the spectrum was taken in MeOH - NaOAc and in MeOH - $\text{AlCl}_3 \cdot \text{HCl}$, respectively. These facts indicated that the isoflavone possessed both 7- and 5-OH. From the observations described above, structure of this compound was elucidated to be 6-chloro-3',4',5,7-tetrahydroxy- or 8-chloro-3',4',5,7-tetrahydroxyisoflavone. Through the long range selective proton decoupling (LSPD) experiments of 3, signals at δ 146.5 and 147.2 were assigned to C-3' and C-4', respectively and δ 155.1 was assigned to C-8a position because this signal was simplified by the irradiation at δ 8.00 (2-H) and δ 162.0* and 162.7* were assigned to C-5 and C-7 (*exchangeable). When a singlet at δ 6.37 (1H, s, 6-H or 8-H) was irradiated, it was observed that both of the signals at δ 162.0 and 162.7 (C-5 and C-7) was simplified to be singlets (Fig. 3). From these observations the singlet at δ 6.37 was assigned to 6-H and the structure of this compound was concluded to be 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3). ^{13}C NMR assignments of 3 are accomplished as follows: 155.0 (C-2), 125.3 (C-3), 182.3 (C-4), 106.9 (C-4a), 162.0 (C-5 or C-7), 100.8 (C-6), 162.7 (C-7 or C-5), 99.7 (C-8), 155.1 (C-8a), 123.7 (C-1'), 117.7 (C-2'), 146.5 (C-3'), 147.2 (C-4'), 116.6 (C-5') and 122.1 (C-6').

Antitumor Activity Tests of 4',7,8-Trihydroxyisoflavone (1)

Antitumor activities of 1 are shown in Table 2.

Table 2. Antitumor activity of 4',7,8-trihydroxyisoflavone (1).

Tumor	Inoculum size and mice	Dose (mg/kg/day)	MSD	ILS (%)
IMC carcinoma	1×10^5 cells/CDF ₁	—	14.7	0
		25	17.7	20
		100	20.0	36
S180	1×10^5 cells/ICR	—	11.0	0
		25	14.7	33
		100	26.3	139
P388 leukemia	1×10^5 cells/CDF ₁	—	9.0	0
		25	9.6	7
		100	10.0	11
P388/ADM leukemia	1×10^5 cells/CDF ₁	—	9.5	0
		25	10.0	5
		100	10.0	5

Discussion

A novel antibiotic, 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3) was isolated from the cultured broth of *Streptomyces* sp. OH-1049 together with 4',7,8-trihydroxyisoflavone (1) and 3',4',7-trihydroxyisoflavone (2). Compounds 1~3 are attributed to 8-hydroxy- and 3'-hydroxydaidzein and 8-chloroorobol, respectively.

Though compound 1 was synthesized previously²⁾, this is the first report of its isolation from the natural source. Compound 2 was previously isolated from the heartwood of *Machaerium villosum* (Leguminosae)¹⁾, whereas this is the first report of its isolation as a fermentation product. Also, this is the first report of the antioxidant activities of compounds 1 and 2.

Compound 3 is a novel isoflavonoid containing a chlorine atom in the molecule. The only known chlorinated isoflavonoids previously isolated are 6-chlorogenistein (6) and 6,3'-dichlorogenistein (7) which are metabolites of *Streptomyces griseus* grown in media containing soybean meal¹⁰⁾.

Compound 1 was synthesized and its antitumor activity was tested. As indicated in Table 2, 1 showed remarkable ILS on S180 bearing mice and slight ILS on IMC carcinoma transplanted mice, but 1 showed no ILS on P388 or P388/ADM leukemia bearing mice.

α -Tocopherol has been proposed for treatment of the cardiotoxicity caused by doxorubicin¹¹⁾. The mechanism of this protection is unknown, but it has been postulated that antioxidation is involved. Since compound 1 not only possesses an antioxidant activity but also possesses antitumor activity, it could play an important role in the treatment of tumors.

We are now investigating further the biological activities of these compounds and their related compounds. The results will be reported elsewhere.

Acknowledgment

This work was supported in part, by Grants-in-Aid from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture, Japan, and by funds from Japan Keirin Association.

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ANTIPROLIFERATIVE EFFECTS OF SYNTHETIC AND NATURALLY
OCCURRING FLAVONOIDS ON TUMOR CELLS OF THE HUMAN
BREAST CARCINOMA CELL LINE, ZR-75-1

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ABSTRACT

An examination was made of the effects of 21 synthetic and naturally occurring flavonoids on the in vitro growth of cells of the human breast carcinoma, ZR-75-1. In all cases, antiproliferative effects were noted, with an IC₅₀ ranging from 2.7 to 33.5 µg/ml, except for the isoflavonoid, daidzin (IC₅₀ > 50 µg/ml). No significant structure-activity relationship among the compounds could be found. Flavone, 6-hydroxyflavone and 4',5,7-trihydroxyflavone (apigenin) were the most potent with IC₅₀ of 2.7, 3.4, and 3.5 µg/ml, respectively. The flavonoid effects observed here were not due to cytostatic action alone, since cell death was found to increase dose-dependently, according to the results of a dye exclusion test.

INTRODUCTION

It has been suggested that phenolic compounds in a fiber-rich diet, such as isoflavonic phytoestrogens and their metabolites, may

provide protective action against breast cancer (Adlercreutz *et al.*, 1982; Adlercreutz, 1984). Indeed, plant flavonoids have been shown to possess anticancer or anticarcinogenic activity against certain experimental tumors (Edwards *et al.*, 1979; Gassady *et al.*, 1988; Verma *et al.*, 1988). Recent reports indicate the potential use of synthetic flavonoid, such as flavone acetic acid (NSC 347512) in warding off certain solid tumors (Bissery *et al.*, 1988; Corbett *et al.*, 1986; Plowman *et al.*, 1986; Zaharko *et al.*, 1986). However, little research has been directed to the assessment of the direct effects of flavonoids on human breast cancer growth either in vivo or in vitro. The anti-cancer and possibly antiestrogenic properties of these compounds and the epidemiological finding that breast cancer incidence is lower in areas where vegetarian or semi-vegetarian diets are consumed (Dunn, 1975; Miller, 1977; Wynder, 1980) prompted us to examine the effects of various synthetic and naturally occurring flavonoids on the in vitro growth of cells from the human breast carcinoma cell line, ZR-75-1 (Engel *et al.*, 1987).

MATERIALS AND METHODS

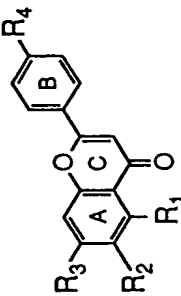
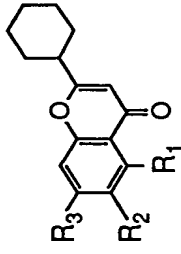
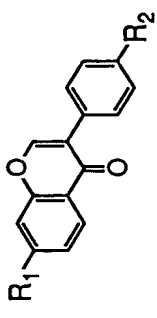
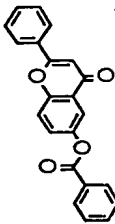
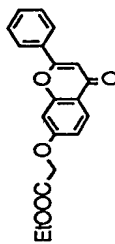
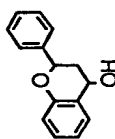
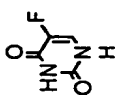
Materials

Flavonoids used in the present study were presented in Table 1. These were synthesized in our Laboratory as follows. Hydroxyflavones (F-1, F-2, and F-3) were prepared by the general method of Baker (Baker, 1933) from the corresponding dihydroxyacetophenones, benzoic anhydride, and sodium benzoate. 2-Cyclohexylchromones (HF-1, HF-2, and HF-3) were obtained by Rh-Al₂O₃ catalyzed hydrogenation of the corresponding hydroxyflavones. Flavanol was obtained by hydrogenation

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Table 1 Structures of flavonoids examined in the present study.

																											
R ₁	R ₂	R ₃	R ₄	IC ₅₀ (μg/ml)				IC ₅₀ (μg/ml)				IC ₅₀ (μg/ml)															
F-0	H	H	H	H	H	H	2.7																				
(flavone)																											
F-1	OH	H	H	H	H	H	4.5	HF-1	OH	H	H	4.0	IF-1	OH	H	21.0											
F-2	H	OH	H	H	H	H	3.4	HF-2	H	OH	H	6.0	IF-2	OH	OH	15.3											
F-3	H	H	OH	H	H	H	33.5	HF-3	H	H	OH	24.0	IF-3	OMe	H	12.7											
F-4	OMe	H	H	H	H	H	13.2	HF-4	H	H	OMe	13.0	IF-4	glucose	OH	>50											
F-5	H	OMe	H	H	H	H	4.3						IF-5	OMe	OMe	11.2											
F-6	H	H	OMe	OH			12.5																				
F-7	OH	H	OH	OH			3.5																				
(apigenin)																											
				F-8								F-9								SF-1							
				IC ₅₀ 21.0 (μg/ml)				IC ₅₀ 22.0				IC ₅₀ 28.0				IC ₅₀ 7.9				IC ₅₀ 0.02							

of flavone (F-0) as a main product. 8-Hydroxyisoflavone (IF-2) was prepared by Friedel-Craft acylation of resorcinol with phenylacetic acid chloride in the presence of anhydrous $AlCl_3$ according to the procedure of Schuda (Schuda and Price, 1987). 2-Hydroxy-8-methoxy-2,3-dihydroisoflavone (SF-1) was prepared from methylation of the corresponding 2,3-dihydroisoflavone, which was obtained as a minor product in the synthesis of compound (IF-2). Methoxy flavonoids (F-4, F-5, F-6, HF-4, and IF-2) were prepared by methylation of the corresponding hydroxyflavonoids. These synthetic flavonoids were identified by spectroscopic and analytical data or by a comparison with literature data. Apigenin (F-7) and 5-fluorouracil were purchased from Sigma Chemical Co., St. Louis, MO.

RPMI 1640 medium and fetal calf serum were obtained from Difco Laboratory, New York. Penicillin and streptomycin were from Meiji Seika, Pharm.Co., Japan. All other reagents were of the best available grade.

Cell culture

ZR-75-1 human breast carcinoma cells (Engel *et al.*, 1978) were cultured at 37°C under 5 % CO_2 -humidified air in a RPMI 1640 medium (GIBCO) containing 10 % fetal calf serum (GIBCO), 100 units/ml of penicillin and 100 μ g/ml of streptomycin. The procedure for cell counting is as follows: remove medium, rinse monolayer with fresh 0.25 % trypsin and allow culture to stand at room temperature for 5-11 min. Add fresh medium, aspirate and dispense the suspension into new flasks. Single cells were washed with fresh medium 3-times and resuspended in the medium at a concentration of 2×10^5 cells /ml. Cell viability following trypsinization as determined by a dye

exclusion test using 0.16 % trypane blue was found to exceed 95 %. One ml of the cell suspension was placed in each well of a 24-well plastic plate followed by adding 10 μ l of the test solution dissolved in ethanol, bringing the final concentration of each flavone to 1.0, 10.0 or 50.0 μ g/ml. Ten μ l of ethanol were added to a control well. The effects of 5-fluorouracil at concentrations of 0.01, 0.1, and 1.0 μ g/ml were also examined. Living cell number was counted by the trypane blue dye exclusion test.

RESULTS AND DISCUSSION

Flavonoids inhibited cell proliferation in a dose-dependent fashion, as evident from Fig.2. In most cases, the effects were clearly apparent after 2 days of treatment and more pronounced at 3 days but was not significant at the first day. The flavonoid concentrations required to inhibit exponential cell growth by 50 % (IC_{50}) ranged from 2.7 to 33.5 μ g/ml, except for the glycosylated isoflavonoid, daidzin (IF-4, $IC_{50} > 50$ μ g/ml, Table 1). No structure-activity relationship among the flavonoids could be clearly discerned. Flavone (F-0), 6-hydroxyflavone (F-2), and 4',5,7-trihydroxyflavone (F-7, apigenin) were the most inhibitory, with IC_{50} of 2.7, 3.4, and 3.5 μ g/ml, respectively. These effects of flavonoids were not due only to the cytostatic activity of the compounds, since cell death was found to increase dose-dependently, according to the results of a trypane blue dye exclusion test. As reported for flavone acetic acid (Axelson *et al.*, 1982), there is the possibility that these flavonoids cause extensive DNA strand breakage. In addition, if there is antiestrogenic action of isoflavonoids

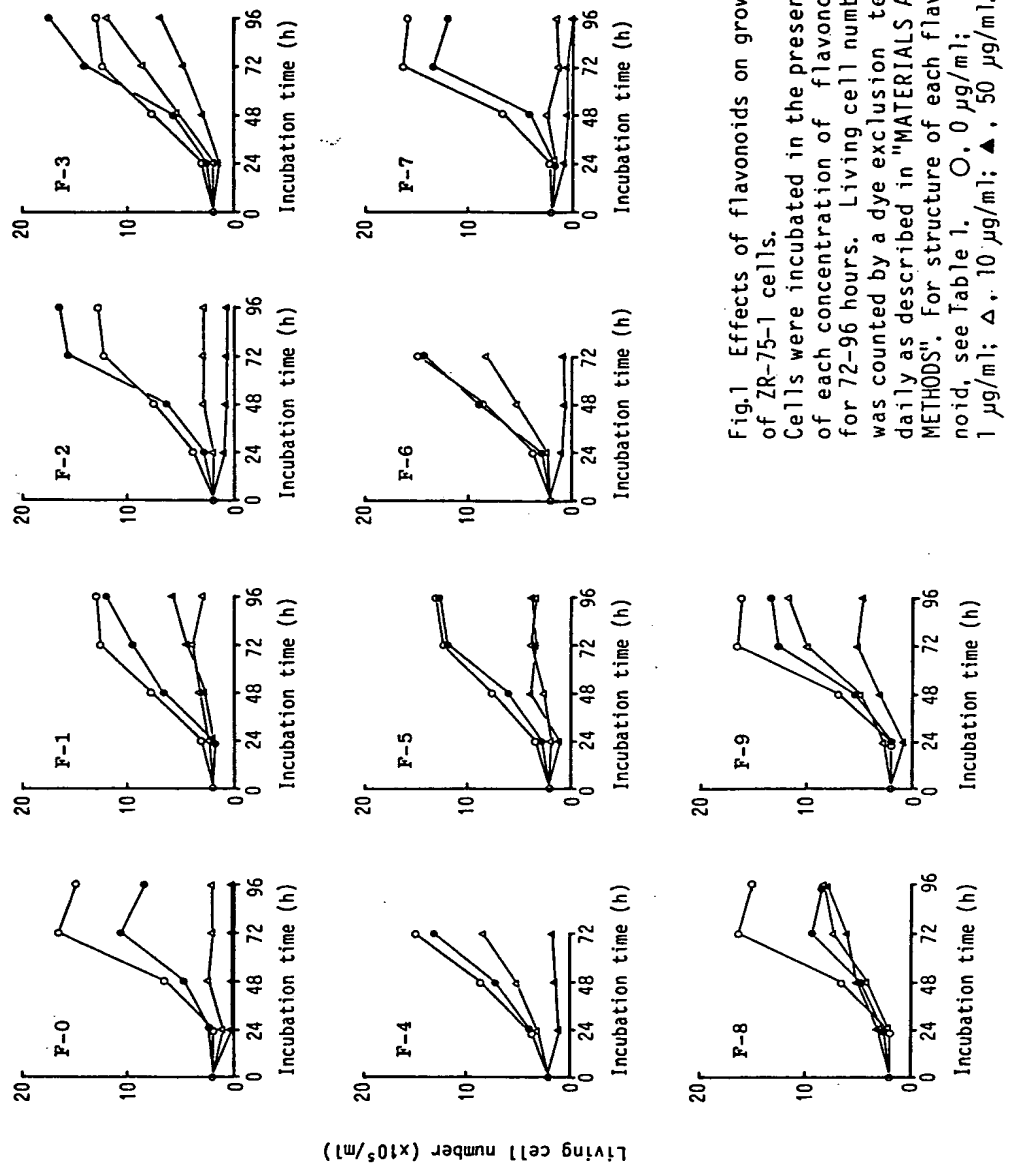


Fig.1 Effects of flavonoids on growth of ZR-75-1 cells. Cells were incubated in the presence of each concentration of flavonoid for 72-96 hours. Living cell number was counted by a dye exclusion test daily as described in "MATERIALS AND METHODS". For structure of each flavonoid, see Table 1. O, 0 µg/ml; Δ , 10 µg/ml; \bullet , 50 µg/ml.

daily as described in "MATERIALS AND METHODS". For structure of each flavonoid, see Table 1. O, 0 μ g/ml; Δ , 1 μ g/ml; \square , 10 μ g/ml; \blacktriangle , 50 μ g/ml.

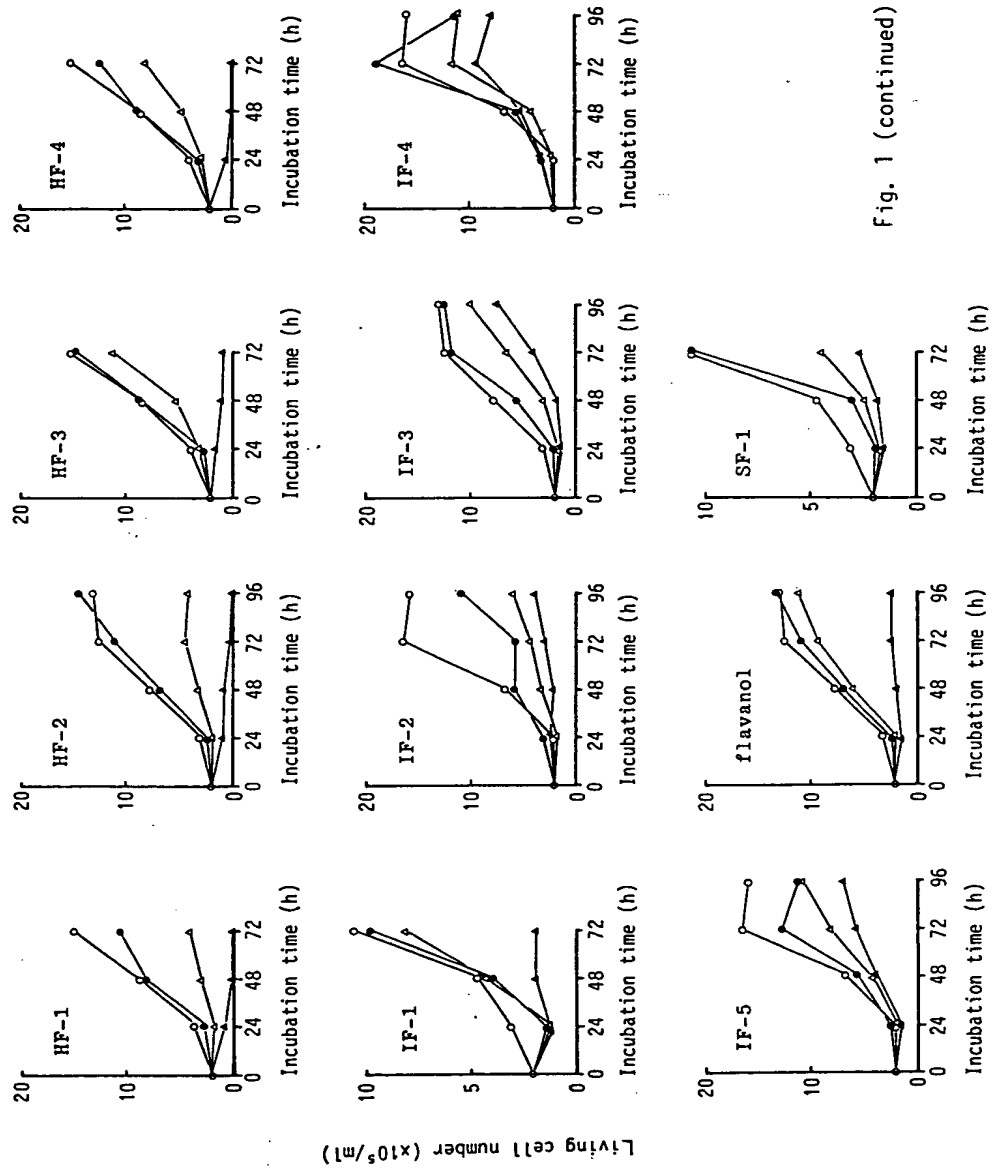
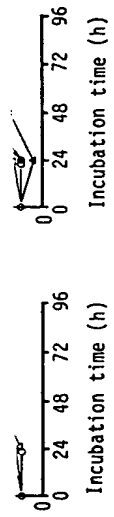


Fig. 1 (continued)

(Adlercreutz *et al.*, 1982; Axelson *et al.*, 1982), this would contribute partially to their inhibitory effects on breast carcinoma cell growth. But this point will require further study for confirmation.

The flavonoid concentrations used in this study to inhibit 50 % cell proliferation (2.7-33.5 $\mu\text{g/ml}$) were a number of orders higher than that of the established anticancer agent, 5-fluorouracil (Table 1). The potency of these flavonoids to inhibit breast carcinoma cell growth is essentially the same as that reported by Edwards *et al.* in other cancer cell systems (Edwards *et al.*, 1979). Thus, in spite of their occasional activity, these compounds as anti-tumor agents do not warrant additional study. However, from our preliminary study, it was noted that the urinary excretion rates of certain isoflavonoids reached 5 $\mu\text{g/h}$ following the consumption of certain leguminous foods or galenicals in humans. This was accompanied by increase in the blood concentrations of these isoflavonoids. Other studies have indicated the presence of isoflavonoids in the biofluids of humans (Bannwart *et al.*, 1984) and nonhuman primates (Adlercreutz *et al.*, 1986). These observations and the findings of the present study support the epidemiological importance in the prophylaxis of breast cancer of certain flavonoids of plant origin, either in their present form or as metabolites produced through the action of intestinal bacteria.

ACKNOWLEDGMENTS

Daidzein (IF-2), daidzin (IF-4), and dimethyldaidzein (IF-5) were kindly provided by Dr.K.Watanabe and Dr.K.Takeya (Tokyo College of Pharmacy). We thank Fujirebio Inc., Japan, for the financial support, and Mrs.S O'hara for her secretarial assistance.

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Vitamin B₁₂ Production by *Citrobacter freundii* or *Klebsiella pneumoniae* during Tempeh Fermentation and Proof of Enterotoxin Absence by PCR

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The influence of some fermentation parameters on vitamin B₁₂ formation by strains of *Citrobacter freundii* and *Klebsiella pneumoniae* isolated from Indonesian tempeh samples during tempeh fermentation was investigated. A decrease in fermentation temperature from 32 to 24°C led to a decrease in vitamin B₁₂ formation. Inoculation of soybeans with different numbers of cells of *C. freundii* at the beginning of solid-substrate fermentation showed that only the velocity of vitamin formation and not the final amount of vitamin formed depended on the number of cells. The addition of cobalt and 5,6-dimethylbenzimidazole increased the vitamin B₁₂ content of tempeh. Nevertheless, levels of incorporation of the two precursors into the vitamin B₁₂ molecule were very low. Neither *C. freundii* nor *K. pneumoniae* possessed the genes encoding the enterotoxins Shiga-like toxin SLT IIA, heat-labile enterotoxin LT I_h, and heat-stable enterotoxin ST I_h, as indicated by PCR. This result supports the suggested use of these two strains to form vitamin B₁₂ during tempeh fermentation in Indonesia.

Tempeh (tempe kedelai) is a traditional fermented soybean food in Indonesia, where it serves as a cheap basic foodstuff in the nutrition of the Indonesian population. It is produced by cooking and hulling soybeans. After a soaking process, the soybeans are cooked once more. For solid-substrate fermentation (SSF), they are inoculated with molds of *Rhizopus* spp. In the traditional process, soybeans are soaked overnight, and spontaneous bacterial acidification takes place. In modern industrial processes in the Western world, bacterial acidification is replaced by artificial acidification with lactic acid. Tempeh is highly significant because of its high content of amino acids, fatty acids, and vitamins (3, 6, 17, 18, 20, 33). The most important vitamin is vitamin B₁₂, which is normally not found in vegetarian foodstuffs (16) and which is formed by bacteria that accompany the fermentation process (20, 22, 28).

In a screening for vitamin B₁₂-producing bacteria isolated from Indonesian tempeh samples, a strain of *Citrobacter freundii* was found to produce the highest vitamin B₁₂ concentration during the tempeh SSF. A *Klebsiella pneumoniae* strain also formed large amounts of this vitamin (20). Besides vitamin B₁₂, these two strains also formed other water-soluble vitamins, such as riboflavin and vitamin B₆. *C. freundii* also produced vitamin B₁₂ during the soaking of soybeans (8).

Some strains of the family *Enterobacteriaceae* are known to be capable of producing enterotoxins (5, 14, 19). Therefore, we investigated whether the aforementioned *C. freundii* and *K. pneumoniae* strains possess three known genes for enterotoxin production: the genes for Shiga-like toxin SLT IIA (30), for heat-labile enterotoxin LT I_h (34), and for heat-stable enterotoxin ST I_h (24).

The aim of the present work was to investigate the influence of fermentation parameters and additions on vitamin B₁₂ formation. Furthermore, it was important to know whether the tested *C. freundii* and *K. pneumoniae* strains possess genes

encoding enterotoxins, because the intention is to use the strains for tempeh fermentation.

MATERIALS AND METHODS

Microorganisms. The mold *Rhizopus oligosporus* (isolate Tebo), which was isolated from Indonesian tempeh (17) and which provides high yields of water-soluble vitamins (20), was used for tempeh fermentation. *C. freundii* (isolate 259) and *K. pneumoniae* (isolate 274) were also added at the beginning of the fermentation process because they had been characterized as producing high levels of vitamin B₁₂ during SSF (20).

Microorganisms isolated from tempeh samples which had been soaked according to the traditional process in our laboratory were identified on the basis of the key to the genera in *Bergey's Manual of Determinative Bacteriology* (7) and checked against the information in *Bergey's Manual of Systematic Bacteriology* (21, 32).

For PCR analysis, the following reference strains were used: *Escherichia coli* C600 W34 (SLT IIA⁺), *E. coli* 2348/I (SLT IIA⁻), *E. coli* O:128H⁻ (LT⁻ ST⁺), *E. coli* O:6H⁻ (LT⁺ ST⁺), and *E. coli* G 1253 (LT⁺ ST⁻); they were kindly provided by H. Karch, Institut für Hygiene und Mikrobiologie, University of Würzburg, Würzburg, Federal Republic of Germany. *E. coli* C600 EWD 299 (LT⁺ ST⁻) and *E. coli* HB101 (SLM 004 (LT⁻ ST⁺)) have been described by Moseley et al. (23) and were kindly provided by them.

Process of tempeh fermentation. The modern tempeh fermentation process was carried out in the standardized way as developed by Hering et al. (17). The beans (300 g, wet weight) were inoculated with a spore suspension (1.8 ml) of *R. oligosporus* Tebo (10⁶ spores/ml of 0.9% NaCl⁻¹ corresponding to 6 × 10³ spores/g of beans⁻¹). In fermentations with *C. freundii* or *K. pneumoniae* 1.8 ml of a cell suspension (10⁶ cells/ml of 0.9% NaCl⁻¹, corresponding to 6 × 10⁴ cells/g of beans⁻¹) was added. After that, the beans were fermented at 32°C for 34 h.

The traditional fermentation process was carried out as

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TABLE 1. Nucleotide sequences of primer pairs and PCR conditions used for the amplification of enterotoxin genes

PCR primer pairs and PCR conditions used for the amplification of enterotoxin genes								
Primer	Nucleotide sequence	PCR conditions						Expected size of fragment (bp)
		Denaturing		Annealing		Extension		
		°C	s	°C	s	°C	s	
SLT IIA start	5'-CCCGGATCCATGAAGTGTATATTATTTAAATGG-3'	94	45	53	60	72	60	960
SLT IIA stop	5'-CCCGAATTCTTATTTACCCGTTGTATATAAAA-3'							
LT 1h start	5'-GCGTTACTATCCTCTCTATG-3'	94	40	49	60	72	45	320
LT 1h stop	5'-ATTGGGGGTTTATTATTCC-3'							
ST 1h start	5'-TCCCTCAGGATGCTAAAC-3'	94	30	47	60	72	30	244
ST 1h stop	5'-GCAACAGGTACATACGTT-3'							

described by Baumann et al. (3) with *C. freundii* only. Soybeans (one part) were washed and cooked with demineralized water (three parts) for 30 min. After a period of cooling down, the soaking water was inoculated with *C. freundii* (3×10^7 cells ml of soaking water⁻¹), and the beans were soaked at 30°C for 15 h. After separation of the beans and soaking water, the beans were hulled, cooked again for 30 min, surface dried, and divided into two portions. One portion was autoclaved before SSF with the *Rhizopus* sp., and the other portion was not sterilized.

The portion of the artificially soaked soybeans and the two portions of the traditionally soaked soybeans were divided into three parts each. One part was inoculated with the *Rhizopus* sp. and *C. freundii*, the second part was inoculated with the *Rhizopus* sp. only, and the third part was inoculated neither with the *Rhizopus* sp. nor with *C. freundii* (control).

As five fermentations under the same conditions had shown a maximal relative standard deviation of $\leq 9.2\%$, we did not replicate each fermentation.

Variation of fermentation parameters. For analyzing the influence of incubation temperature, tempeh fermentations were carried out at 24, 28, and 32°C. The influence of different numbers of bacterial cells on vitamin B₁₂ formation was investigated by inoculating soybeans with suspensions of 10^3 to 10^8 cells ml⁻¹ (equivalent to 7×10^0 to 7×10^5 cells g of beans⁻¹).

Cobalt(II)-sulfate-heptahydrate and 5,6-dimethylbenzimidazole at a concentration range of 0 to 400 mg of bacterial suspension liter⁻¹ (equivalent to 0.135 to 2.7 µg g of beans⁻¹) were added to the bacterial suspension, with which the beans were inoculated before SSF with the *Rhizopus* sp. The yield was determined by dividing the number of vitamin B₁₂ molecules formed by the number of precursor molecules added.

Determination of bacterial growth in tempeh. The growth of bacteria in tempeh was determined as described by Hausler (15). Bacteria were spread out on plate count agar (E. Merck, Darmstadt, Federal Republic of Germany) supplemented with cycloheximide (100 mg liter⁻¹).

Vitamin B₁₂ analysis. Vitamin B₁₂ analysis was done with a microbiological assay as described by Okada et al. (28); this assay is considered to distinguish between different forms of corrinoids: physiologically active vitamin B₁₂ (cobalamins), which can be used by humans, and analogous forms, which cannot be so used. All vitamin B₁₂ assays were executed in triplicate at two different dilutions, each analyzed eight times. The maximal relative standard deviation was $\leq 7.5\%$.

PCR analysis. For PCR analysis, *C. freundii* (259), *K. pneumoniae* (274), and the reference strains were cultivated in 25 ml of LB medium, containing (grams liter⁻¹) tryptone (10), yeast extract (5), and NaCl (10), (pH 7.5), at 27°C on a shaker

(model G 76 Gyrotory water bath shaker; New Brunswick Scientific Co., Edison, N.J.) for 15 h at 200 rpm. In the case of SLT IIA, ampicillin (100 ng ml⁻¹; Sigma Chemical Co., St. Louis, Mo.) was added to one of two parallel cultures because the formation of SLT IIA is sometimes enhanced in the presence of this antibiotic.

The oligonucleotides used as primers for the amplification of a part of the SLT IIA gene were described by Gunzer et al. (13). The primers used for the amplification of a part of the LT 1h gene were derived from the B subunit (34). For the amplification of a part of the ST 1h gene, the primers were derived from the ST sequence (24). The nucleotide sequences of the primer pairs and the PCR conditions used for the amplification of the three genes are listed in Table 1. All primers, which were purified by high-performance liquid chromatography, were purchased from MWG-Biotech (Ebersberg, Federal Republic of Germany). The PCR analysis was done as described by Schmidt et al. (30).

RESULTS

Influence of fermentation temperature. With both bacterial strains, fermentations performed at 32°C resulted in the highest vitamin B₁₂ content (Table 2). Lowering the incubation temperature from 32 to 28°C and from 32 to 24°C resulted in a decrease in vitamin B₁₂ content in tempeh. A further effect of the lowered temperature was a prolongation of the fermentation time for the *Rhizopus* sp. from 34 h (32°C) to 50 h (28°C) and to 67 h (24°C). *C. freundii* formed higher vitamin B₁₂ concentrations than *K. pneumoniae* at all temperatures investigated.

Influence of cobalt and 5,6-dimethylbenzimidazole. The addition of cobalt resulted in an increase in vitamin B₁₂ formation (Fig. 1). Fermentations with *K. pneumoniae* which were supplemented with low concentrations of cobalt(II)-sulfate-heptahydrate (0 to 100 mg liter⁻¹) especially showed a steep increase in vitamin B₁₂ content from 73 to 170 ng g of dry weight⁻¹. The increase in vitamin B₁₂ content in fermentations

TABLE 2. Formation of vitamin B₁₂ by *C. freundii* (259) and *K. pneumoniae* (274) at different fermentation temperatures^a

Temp (°C)	Cyanocobalamin (ng g of dry wt ⁻¹)	
	<i>C. freundii</i>	<i>K. pneumoniae</i>
24	114	74
28	130	95
32	152	135

^a The maximal relative standard deviation was $\leq 7.5\%$.

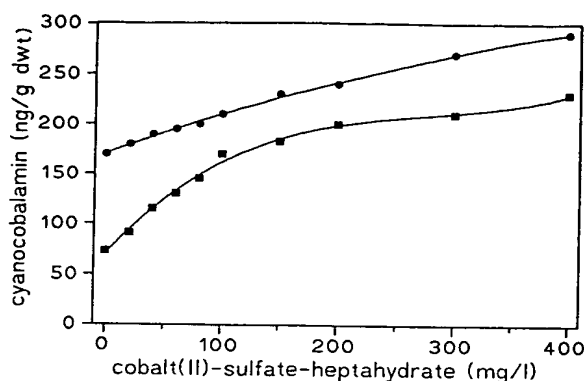


FIG. 1. Formation of vitamin B₁₂ (cyanocobalamin) during tempeh fermentations by *C. freundii* (259) (●) and *K. pneumoniae* (274) (■) after the addition of cobalt(II)-sulfate-heptahydrate. The maximal relative standard deviation was $\leq 7.5\%$. dwt, dry weight.

with *C. freundii* developed in a straight line with increasing cobalt concentrations, and vitamin B₁₂ content reached a maximal level of 290 ng g of dry weight⁻¹. Nevertheless, the incorporation of the precursor was not really effective, as the resulting yields were very low and reached a maximal value of only 0.014 (data not shown).

The addition of 5,6-dimethylbenzimidazole also resulted in an increase in vitamin B₁₂ formation, especially at low concentrations (0 to 100 mg liter⁻¹) (Fig. 2). In contrast to fermentations with *K. pneumoniae*, fermentations with *C. freundii* showed a further increase with concentrations of up to 300 mg liter⁻¹. The yields for the incorporation of 5,6-dimethylbenzimidazole into vitamin B₁₂ were also very low and reached a maximal value of only 0.013 (data not shown). The numbers of cells were not influenced by the addition of the two precursors and were comparable in all fermentations.

Influence of inoculation of soybeans with different numbers of cells of *C. freundii*. The effect of inoculum size was investigated with the most effective vitamin B₁₂-producing organism, *C. freundii*. Although the initial numbers of cells differed from 10¹ to 10⁶ (g of wet weight⁻¹), after 27 h all cell numbers approached 10⁹ to 10¹⁰ (g of wet weight⁻¹) (Fig. 3A) as a result of more rapid growth rates for small inocula. After 27 h

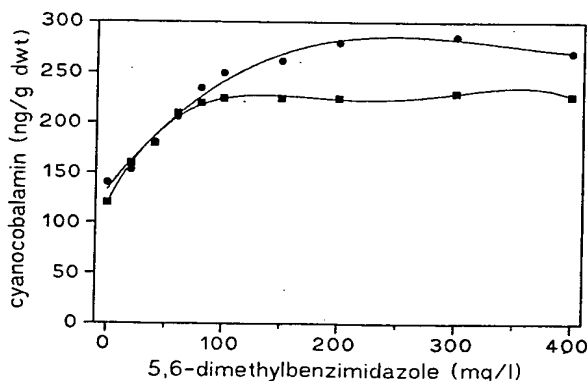


FIG. 2. Formation of vitamin B₁₂ (cyanocobalamin) during tempeh fermentations by *C. freundii* (259) (●) and *K. pneumoniae* (274) (■) after the addition of 5,6-dimethylbenzimidazole. The maximal relative standard deviation was $\leq 7.5\%$. dwt, dry weight.

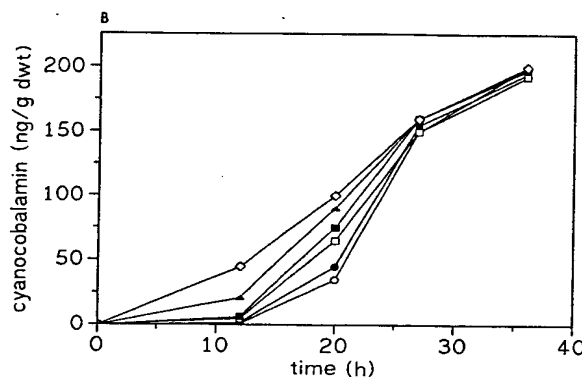
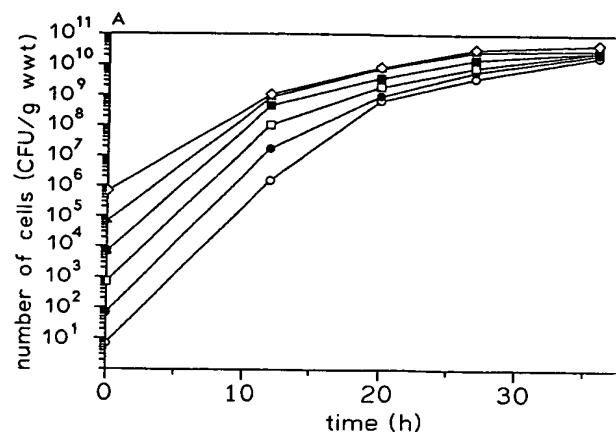


FIG. 3. (A) Numbers of cells of *C. freundii* (259) during ten fermentation after inoculation of soybeans with different numbers of cells. ww, wet weight. (B) Dependence of the formation of vitamin B₁₂ (cyanocobalamin) on the inoculation of soybeans with different numbers of cells of *C. freundii* (259). The maximal relative standard deviation was $\leq 7.5\%$. dwt, dry weight. Symbols (for both panels) 10⁶; ▲, 10⁷; ■, 10⁸; □, 10⁹; ●, 10⁴; ○, 10³.

the vitamin B₁₂ contents of the different fermentations reached nearly the same range of 150 to 160 ng g of dry weight⁻¹ in accordance with the increase in the cell numbers (Fig. 3B).

Influence of the preparatory treatment of soybeans for tempeh on vitamin B₁₂ formation. Different techniques were compared with regard to vitamin B₁₂ formation during SSF. The fermentation carried out with lactate soaking of soybeans and in which the soybeans were inoculated with the *Rhizopus* sp. and *C. freundii* for SSF resulted in the highest vitamin B₁₂ level (Table 3). Tempeh prepared with traditionally soaked (soaking with water) soybeans inoculated with *C. freundii* soybeans which were also inoculated with the *Rhizopus* sp. and *C. freundii* before SSF showed low vitamin B₁₂ levels.

There was also a difference between tempeh prepared with autoclaved and nonautoclaved soybeans. In a sample prepared with autoclaved soybeans, only *C. freundii* could be detected after SSF with *C. freundii*. In a sample prepared with nonautoclaved soybeans inoculated with *C. freundii*, *Bacillus cereus* was also isolated. Tempeh prepared with traditionally soaked (with *C. freundii*), nonautoclaved soybeans inoculated with *Rhizopus* sp. and *C. freundii* for SSF contained higher vitamin B₁₂ concentrations than tempeh prepared with traditionally soaked (with *C. freundii*), nonautoclaved soybeans inoculated with only the *Rhizopus* sp.

TABLE 3. Formation of vitamin B₁₂ (cyanocobalamin) and numbers of cells during tempeh fermentations carried out under various conditions^a

Method of fermentation ^b	Cyanocobalamin (ng g of dry wt ⁻¹) produced	Total plate count of <i>C. freundii</i> on plate count agar (10 ⁹)
<i>R. oligosporus</i> + <i>C. freundii</i>		
Lactate soaking	53.9	50.0
Autoclaving	48.8	35.0
No autoclaving	33.0	50.0
<i>R. oligosporus</i>		
Lactate soaking	ND ^c	ND
Autoclaving	ND	ND
No autoclaving	23.2	5.0
Control		
Lactate soaking	ND	ND
Autoclaving	ND	ND
No autoclaving	3.5	0.1

^a The maximal relative standard deviation was $\leq 7.5\%$.^b For no autoclaving, beans were soaked overnight with *C. freundii* and cooked but not autoclaved after soaking. For autoclaving, beans were soaked overnight with *C. freundii*, cooked, and autoclaved. For lactate soaking, beans were soaked with lactate and autoclaved after soaking and cooking.^c ND, not detectable.

Control soybeans (without any inoculation before SSF) soaked in the traditional way (with *C. freundii*) and not autoclaved also contained vitamin B₁₂. In these beans, *C. freundii*, *B. cereus*, and *Micrococcus luteus* could be detected. Fermentations carried out with traditionally soaked and with lactic acid-soaked beans, both of which were autoclaved after soaking, resulted in no vitamin B₁₂ when inoculation for SSF was done with the *Rhizopus* sp. alone.

Detection of the enterotoxin-encoding genes by PCR. Tests for the three enterotoxin (SLT IIA, LT I_h, and ST I_h) genes were negative for *C. freundii* and for *K. pneumoniae*. Figure 4 shows the test for the enterotoxin ST I_h gene.

DISCUSSION

The fact that vitamin B₁₂ production by *C. freundii* or *K. pneumoniae* is increased by raising the fermentation temperature is in contrast to the amounts of free amino acids (2, 6) and the content of γ -linolenic acid (17) in tempeh, which are improved by lowering the fermentation temperature from 32 to 24°C. As vitamin B₁₂ production is coupled to the growth of the bacteria (11), it is obvious why more vitamin B₁₂ is formed by the bacteria at 32 than at 24°C.

The increase in vitamin B₁₂ concentrations with the addition

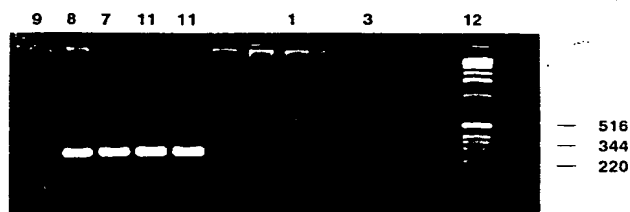


FIG. 4. PCR products of the gene for heat-stable enterotoxin ST I_h. 1, *C. freundii* 259; 3, *K. pneumoniae* 274; 7, *E. coli* O:128H⁻ (LT⁻ ST⁺); 8, *E. coli* O:6H⁻ (LT⁺ ST⁺); 9, *E. coli* G 1253 (LT⁺ ST⁻); 11, *E. coli* HB101 SLM 004 (LT⁻ ST⁺); 12, ladder (in base pairs).

of cobalt shows that the cobalt content in soybeans is suboptimal for vitamin B₁₂ production by *C. freundii* or *K. pneumoniae*. Although the addition of cobalt and 5,6-dimethylbenzimidazole leads to an increase in vitamin B₁₂ production by both strains, their incorporation is poor, as shown by the low yields. Moreover, supplementation with these precursors is not necessary, as the vitamin B₁₂ concentration produced in tempeh by either of these two strains would be high enough to meet the daily requirement of an adult person.

Inoculation of soybeans with different numbers of cells of *C. freundii* showed that vitamin B₁₂ production is correlated with the growth of the bacterium. This was also confirmed for *Propionibacterium freudenreichii* (11). The rate of vitamin B₁₂ production by *C. freundii* depended on the number of cells in the inoculum but not the final amount.

The low level of vitamin B₁₂ in the fermentation with traditionally soaked, nonautoclaved soybeans additionally inoculated with *C. freundii* before SSF can be explained by the existence of a mixed culture in this tempeh sample. In mixed cultures with other bacteria *C. freundii* does not produce as much vitamin B₁₂ as it does in pure cultures, as proved in another fermentation (data not shown).

The appearance of *C. freundii* and *B. cereus* in tempeh prepared with traditionally soaked, nonautoclaved soybeans can be explained by the nonsterile conditions of the tempeh fermentation process, which imitates the process performed in Indonesia. The results show that bacteria which are present during the soaking of soybeans can be transferred to SSF. In Indonesia, this fact is important, as the soybeans are not inoculated with vitamin B₁₂-forming bacteria. In conclusion, vitamin B₁₂ formation only takes place if vitamin B₁₂-forming bacteria that occur by chance are transferred from the soaking stage by handling of the soybeans under nonsterile condition after the second cooking.

Rhizopus spp. have a positive effect on bacterial growth. The reason is the hydrolyzing capacity of the mold, which supplies growth substrates, such as amino acids, for the nutrition of the bacteria (2, 20). Furthermore, protease inhibitors, such as the Bowman-Birk inhibitor, are found in soybeans and can inhibit bacterial serine proteases (4). In contrast, the fungus forms proteases of the aspartate type; these are not sensitive to the inhibitor (1) and therefore can release amino acids in the presence of serine protease inhibitors.

In conclusion, the comparison of the different preparatory treatments of soybeans for SSF shows that the best way of reaching a high vitamin B₁₂ level is lactate soaking of soybeans and inoculation with a vitamin B₁₂-producing bacterium, such as *C. freundii* 259, before SSF.

The family *Enterobacteriaceae* includes many pathogenic organisms, such as *Salmonella typhi*, *Shigella dysenteriae*, *K. pneumoniae*, and *Yersinia pestis*. In addition, strains of *E. coli* and *C. freundii*, which belong to the intestinal flora of humans, can cause diarrhea via the production of enterotoxins (9, 10, 12, 19, 23, 30, 31). Although tempeh contains many members of the family *Enterobacteriaceae* (25–27, 29), no reports exist about diarrhea occurring after the consumption of tempeh. Nevertheless, it had to be determined whether the two strains of *C. freundii* and *K. pneumoniae* used here possess the ability to produce enterotoxins before they could be used for tempeh fermentation. As both strains were negative for three known enterotoxins and as they were originally isolated from Indonesian tempeh, it can now be stated that their use in tempeh fermentation should have no negative effect on tempeh consumers.

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Malonyl Isoflavone Glycosides in Soybean Seeds (*Glycine max* MERRILL)

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The isoflavone constituents in soybean seeds were investigated, and 9 kinds of isoflavone glycosides were isolated from the hypocotyls of soybean seeds. Three kinds were proved to be malonylated soybean isoflavones named 6''-O-malonyldaidzin, 6''-O-malonylglycitin and 6''-O-malonylgenistin by UV, MS, IR and NMR. The malonylated isoflavone glycosides as major isoflavone constituents in soybean seed were thermally unstable, and were converted into their corresponding isoflavone glycosides. All of the isoflavone components produced intensely undesirable taste effects such as bitter, astringent and dry mouth feeling.

Soybeans are known to contain the five isoflavone glycosides, genistin,¹⁾ daidzin,¹⁾ glycitein 7-O-β-D-glucoside (glycitin),²⁾ 6''-O-acetylgenistin³⁾ and 6''-O-acetyldaidzin,⁴⁾ and their corresponding aglycones, daidzein, glycitein and genistein (Fig. 1). It has been reported that these isoflavone compounds possessed antihemolytic,⁵⁾ antioxidative,⁵⁾ antifungal,⁶⁾ oestrogenic⁷⁾ and antitumoral⁸⁾ activities, and

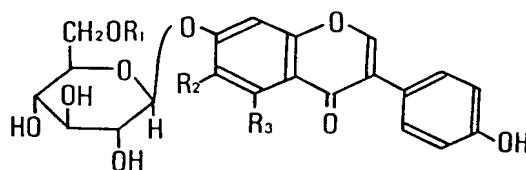
also exhibited undesirable bitter and astringent tastes.^{9,10)} In a previous paper,¹¹⁾ the isolation of a new isoflavone, glycitein 7-O-β-(6''-O-acetyl)-D-glucoside (6''-O-acetylglycitin), was reported, this forming part of one of the components responsible for the undesirable taste characteristics in soybean (Fig. 1). Further investigation suggested that malonyl daidzin and malonyl genistin were likely to occur in soybean seed.¹²⁾ The presence of malonyl genistin has been reported in clover,¹³⁾ and the use of malonyl daidzin from *Pueraria lobata* as an aldose reductase inhibitor has been patented.¹⁴⁾

In this paper, the isolation and chemical structure of the three malonyl isoflavone glycosides, and their taste characteristics are described.

Results and Discussion

Effect of extraction temperature on isoflavone composition

To determine the extraction conditions for



Compound	R ₁	R ₂	R ₃
1 Daidzin	H	H	H
2 Glycitin	H	OCH ₃	H
3 Genistin	H	H	OH
4 6''-O-Malonyldaidzin	COCH ₂ COOH	H	H
5 6''-O-Malonylglycitin	COCH ₂ COOH	OCH ₃	H
6 6''-O-Malonylgenistin	COCH ₂ COOH	H	OH
7 6''-O-Acetyldaidzin	COCH ₃	H	H
8 6''-O-Acetylglycitin	COCH ₃	OCH ₃	H
9 6''-O-Acetylgenistin	COCH ₃	H	OH

Fig. 1. Structures of the Isoflavones in Soybean Seeds.

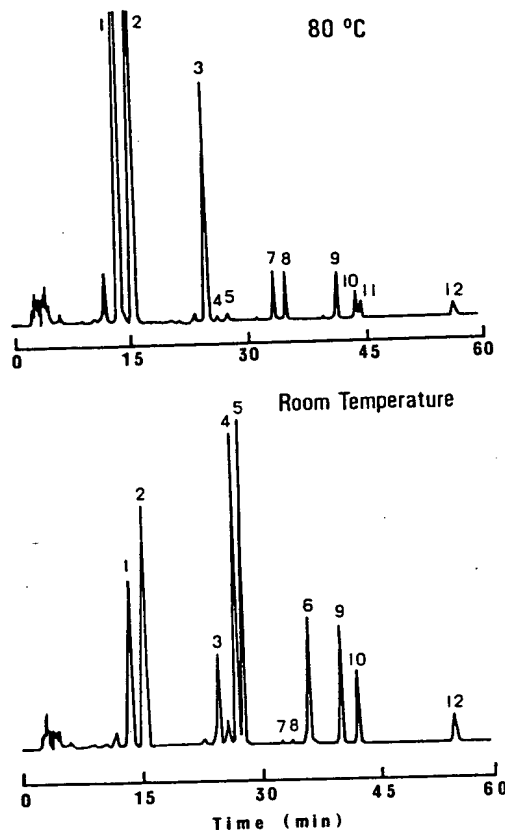


Fig. 2. High-performance Liquid Chromatogram of 70% Ethanol Extracts Obtained from the Hypocotyls of Mature Soybean Seeds at Room Temperature and at 80°C. 1, daidzin; 2, glycitin; 3, genistin; 4, 6''-O-malonyldaidzin; 5, 6''-O-malonylglycitin; 6, 6''-O-malonylgenistin; 7, 6''-O-acetyldaidzin; 8, 6''-O-acetylglycitin; 9, 6''-O-acetylgenistin; 10, daidzein; 11, glycitein; 12, genistein. See the experimental section for HPLC conditions.

isoflavone compounds from soybean seeds, the isoflavone constituents extracted with 70% aqueous ethanol at 80°C and at room temperature were compared by HPLC. As shown in Fig. 2, compounds 4, 5 and 6 were detected as the major constituents in the extract obtained at room temperature, whereas the major constituents in the extract at 80°C were compounds 1, 2 and 3. This suggested that compounds 4, 5 and 6 were thermally unstable, and were converted into compounds 1, 2 and 3. Previously,¹¹⁾ compounds 1, 2, 3, 7, 8 and

9 have been identified as daidzin, glycitin, genistin, 6''-O-acetyldaidzin, 6''-O-acetylglycitin and 6''-O-acetylgenistin by comparing their UV, MS and NMR spectra with published data.²⁻⁴⁾ The isolation of compounds 4, 5 and 6 was effected and their structures determined.

Structures of compounds 4, 5 and 6

Compounds 4, 5 and 6 were isolated from the hypocotyl of soybean seeds (Perikan Co., Ltd.) according to the procedure given in the experimental section.

Mass and UV spectral data for compounds 4, 5 and 6 showed them to be daidzin, glycitin and genistin derivatives, respectively.^{2-4,15)} Prominent $[M+H-86]$ ions due to the loss of malonate were observed in the FAB mass spectra of the three compounds, indicating that these were malonylated isoflavone glycosides.¹⁶⁻¹⁸⁾

Assignments from the ^1H - and ^{13}C -NMR spectra of daidzin, glycitin, genistin, compounds 4, 5 and 6, were established by ^{13}C - ^1H COSY spectra coupled with ^1H - ^1H COSY spectra, and are summarized in Tables I and II. The ^{13}C - and ^1H -NMR spectral data for demalonylated compounds 4, 5 and 6 prepared with 0.5 N aqueous Na_2CO_3 ¹³⁾ were completely consistent with those of daidzin, glycitin and genistin, respectively. The ^{13}C -NMR spectra of compound 4, 5 and 6 showed signals of δ 167.9, 166.8 and 41.4 (COOR, COOH and $-\text{CH}_2-$ in a malonyl group), neither of which were detected in the spectra of their corresponding isoflavone glycosides. The low field methylene protons Ha-6'' and Hb-6'', and the chemical shifts of C-6'' and C-5'' in compounds 4, 5 and 6 when compared with those of daidzin, glycitin and genistin indicated acylation of the sugar moieties at C-6''.¹⁶⁻¹⁸⁾

Thus, the structures of compounds 4, 5 and 6 were deduced to be 6''-O-malonyldaidzin, 6''-O-malonylglycitin and 6''-O-malonylgenistin, respectively. As far as we know, this is the first report of 6''-O-malonylglycitin. The presence of 6''-O-malonylgenistin in clover¹³⁾ and of 6''-O-malonyldaidzin in *Pueraria lobata*¹⁴⁾ has been described before, but not in

Table I. ^1H -NMR DATA FOR ISOFLAVONE GLYCOSIDES FROM THE HYPOCOTYL OF SOYBEAN SEED (400 MHz, in $\text{DMSO}-d_6$, δ_{H})

	Daidzin	Manonyl daidzin	Glycitin	Malonyl glycitin	Genistin	Malonyl genistin
A and C rings						
C-2	8.38 s	8.35 s	8.37 s	8.32 s	8.41 s	8.39 s
C-5	8.05 d (8.8)	8.07 d (8.8)	7.48 s	7.47 s		
C-6	7.14 dd (8.8, 2.4)	7.14 d (8.8, 2.4)			6.47 d (2.5)	6.47 d (2.5)
C-8	7.23 d (2.4)	7.22 d (2.4)	7.32 s	7.31 s	6.72 d (2.5)	6.70 d (2.5)
OMe			3.88 s	3.88 s		
OH					12.92 br	12.94 br
B ring						
C-2' and 6'	7.41 d (8.8)	7.40 d (8.8)	7.41 d (8.8)	7.40 d (8.8)	7.40 d (8.8)	7.39 d (8.8)
C-3' and 5'	6.82 d (8.8)	6.82 d (8.8)	6.82 d (8.8)	6.82 d (8.8)	6.83 d (8.8)	6.83 d (8.8)
OH	9.57 br	9.57 br	9.57 br	9.57 br	9.66 br	9.64 br
Glucose moiety						
C-1"	5.17 d (7.3)	5.14 d (7.3)	5.18 d (7.8)	5.20 d (7.8)	5.06 d (7.3)	5.12 d (7.3)
C-2"-5"	3.15-3.85	3.15-3.80 m	3.15-3.85	3.15-3.80 m	3.15-3.85	3.15-3.80 m
C-6"a		4.41 d (12.0)		4.38 d (12.2)		4.36 d (12.0)
C-6"b		4.11 dd (12.0, 7.1)		4.12 dd (12.2, 6.8)		4.12 dd (12.0, 7.1)
Malonyl C-2"		3.40		3.38		3.38

as daidzin, glycitin, idzin, 6"-O-acetylglycitin by comparing their spectra with published of compounds 4, 5 and structures determined.

Is 4, 5 and 6

and 6 were isolated from soybean seeds (Perikan Co., procedure given in the

ral data for compounds n to be daidzin, glycitin ves, respectively.^{2-4,15} [6] ions due to the loss served in the FAB mass compounds, indicating ylated isoflavone glyco-

the ^1H - and ^{13}C -NMR glycitin, genistin, com-e established by ^{13}C - ^1H ed with ^1H - ^1H COSY narized in Tables I and NMR spectral data for ounds 4, 5 and 6 prepared $^{13}\text{CO}_2$ ¹³) were completely of daidzin, glycitin and The ^{13}C -NMR spectra d 6 showed signals of δ 4 (COOR, COOH and group), neither of which spectra of their corre-glycosides. The low field a-6" and Hb-6", and the " and C-5" in compounds ompared with those of genistin indicated acyla-ties at C-6".¹⁶⁻¹⁸)

s of compounds 4, 5 and be 6"-O-malonyldaidzin, and 6"-O-malonylgenis-ar as we know, this is the -O-malonylglycitin. The onylgenistin in clover¹³) nyldaidzin in *Pueraria* scribed before, but not in

soybean seeds.

Isoflavone accumulation during the development of soybean seeds

The accumulation of daidzin, genistin, glycitin and their corresponding malonylated forms in immature seeds during development followed from between 35 and 60 days after flowering (Fig. 3). Malonylgenistin and the genistin content increased during the late development of the beans, whereas malonyldaidzin and daidzin accumulated throughout the whole period. Minor isoflavone glycosides, malonylglycitin and glycitin, were also detected. The isoflavone content of the mature seed reported in Table III shows that malonylgenistin and malonyldaidzin comprised 66% of the total isoflavones in the beans.

Isoflavone content in mature soybean seeds

The isoflavone content of mature soybean seeds was compared by using two extraction methods. Whole soybean seeds were divided into the seed coat, cotyledon and hypocotyl (containing the plumule and radicle), and each part was extracted at room temperature for 24 hr or at 80°C for 15 hr by a 10-fold volume

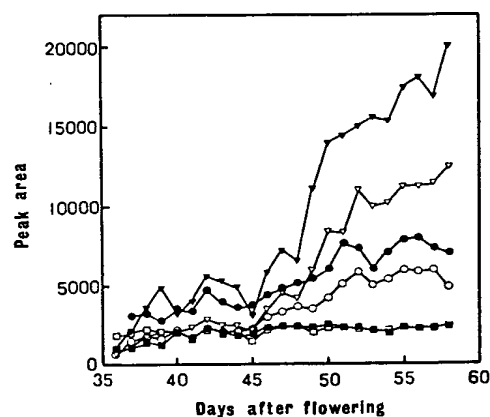


Fig. 3. Isoflavone Accumulation during the Maturation of Maple Arrow Soybean Seeds.

Immature seeds in pods harvested at various stages of maturation after flowering were used as the samples for quantitative analyses. Peak area shows the change in relative amounts of isoflavone glycosides for a certain dry weight. \circ , daidzin; \square , glycitin; ∇ , genistin; \blacksquare , 6"-O-malonyldaidzin; \bullet , 6"-O-malonylglycitin; \blacktriangledown , 6"-O-malonylgenistin.

of 70% aqueous ethanol. As shown in Table IV, the total isoflavone content of the hypocotyl part was 5.5 or 6.0 times higher than that of the cotyledons, and glycitin and its

Table II. ^{13}C -NMR DATA FOR ISOFLAVONE GLYCOSIDES ISOLATED FROM THE HYPOCOTYL OF SOYBEAN (100 MHz, in $\text{DMSO}-d_6$, δ_c)

	Daidzin	Malonyl daidzin	Glycitin	Malonyl glycitin	Genistin	Malonyl genistin
A and C rings						
C-2	153.3	153.3	153.1	153.1	154.6	154.6
C-3	123.7	123.7	123.1	123.1	122.6	122.6
C-4	174.8	174.8	174.3	174.4	180.5	180.5
C-5	127.0	127.1	104.8	104.8	161.7	161.7
C-6	115.6	115.4	147.4	147.4	99.6	99.5
C-7	161.4	161.1	151.1 ^a	151.2 ^a	163.0	162.7
C-8	103.4	103.6	103.6	103.6	94.6	94.6
C-9	157.0	157.0	151.2 ^a	151.2 ^a	157.2	157.2
C-10	118.5	118.6	117.9	117.9	106.1	106.2
B ring						
C-1'	122.3	122.3	122.5	122.6	121.0	121.0
C-2' and 6'	130.1	130.1	130.0	130.0	130.2	130.2
C-3' and 5'	115.0	115.0	114.9	115.0	115.1	115.1
C-4'	157.3	157.3	157.2	157.2	157.5	157.5
OCH_3			55.8	55.9		
Glucose moiety						
C-1''	100.0	99.8	99.7	99.4	99.9	99.5
C-2''	73.2	73.0	73.0	72.9	73.1	73.0
C-3''	76.5	76.2	76.8	76.5	76.4	76.1
C-4''	69.7	69.7	69.6	69.6	69.6	69.6
C-5''	77.2	73.8	77.2	73.8	77.2	73.7
C-6''	60.7	64.1	60.6	64.0	60.6	64.0
Malonyl						
COOR		167.9		168.0		167.9
CH_2		41.4		41.5		41.5
COOH		166.8		167.0		166.9

^a Assignments for these signals within the same column may be interchanged.

Table III. ISOFLAVONE CONTENT IN MAPLE ARROW SOYBEAN SEEDS^a

Compound	Amount ^b (mg)
Daidzin	129.4
Glycitin	20.5
Genistin	96.6
6''-O-Malonyldaidzin	251.0
6''-O-Malonylglycitin	20.0
6''-O-Malonylgenistin	275.5

^a Whole mature seeds were used.

^b per 100 g dry weight.

derivative only occurred in the hypocotyl part of the soybean seed. Isoflavones were absent from the seed coat of soybean. While malonylated isoflavone glycosides were major

constituents in the extract at room temperature, the contents of malonylated isoflavone glycosides in the extract at 80°C decreased significantly, and an increase in all isoflavone glycosides and acetyl isoflavone glycosides, with the exception of 6''-O-acetylgenistin, was observed. The acetyl derivatives may thus have arisen from corresponding malonyl derivatives as artifacts during the work-up procedures, as was pointed out by Horowitz *et al.*¹⁹⁾

Threshold values for the isoflavone components

The threshold value and taste character of each isoflavone component were examined as described in the experimental section. All of the isoflavone components produced intensely undesirable taste characteristics such as bit-

HYPOCOTYL

nistin	Malonyl genistin
54.6	154.6
22.6	122.6
80.5	180.5
61.7	161.7
99.6	99.5
63.0	162.7
94.6	94.6
57.2	157.2
06.1	106.2
21.0	121.0
30.2	130.2
15.1	115.1
57.5	157.5
99.9	99.5
73.1	73.0
76.4	76.1
69.6	69.6
77.2	73.7
60.6	64.0
	167.9
	41.5
	166.9

et at room temperature, ylated isoflavone gly- et at 80°C decreased crease in all isoflavone isoflavone glycosides, 6''-O-acetylgenistin, was ratives may thus have ling malonyl derivatives work-up procedures, as rowitz *et al.*¹⁹⁾

ie isoflavone components e and taste character of onent were examined as rimental section. All of nents produced intensely aracteristics such as bit-

Table IV. CONTENT OF ISOFLAVONE COMPOUNDS IN SOYBEAN SEED^a (mg/100 g)

Compound	Room temperature ^b		80 °C ^c	
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon
Daidzin	320	45	838	145
Glycitin	485	— ^d	1004	—
Genistin	118	80	246	210
6''-O-Malonyl daidzin	423	70	8	3
6''-O-Malonyl glycitin	445	—	11	—
6''-O-Malonyl genistin	144	117	4	—
6''-O-Acetyl daidzin	2	2	57	8
6''-O-Acetyl glycitin	6	—	89	—
6''-O-Acetyl genistin	105	1	39	1
Daidzein	102	33	35	11
Glycitein	—	—	15	—
Genistein	35	48	16	14
Total	2185	396	2362	392

^a Suzuyutaka strain cultivated in Akita Prefecture in 1988.

^b Extraction at room temperature for 24 hr.

^c Extraction at 80 °C for 15 hr.

^d Not detected.

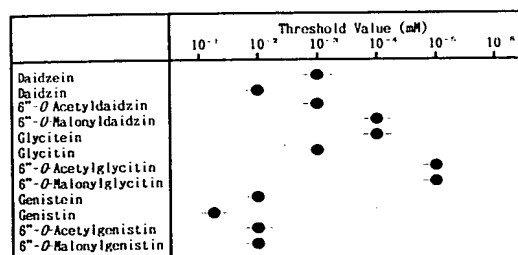


Fig. 4. Threshold Values of the Isoflavones in Soybean Seeds.

terness, astringency and dry mouth feeling. The threshold values for daidzin and its related compounds were in the order of daidzin > daidzein = acetyl daidzin > malonyldaidzin (Fig. 4). Those of the related compounds of glycitin and genistin were in the order of glycoside > aglycone ≥ 6''-O-acyl glycoside, confirming the results by Francis for genistin and malonylgenistin.²⁰⁾ Matsuura *et al.*²¹⁾ have demonstrated that an increase in daidzein and genistein by the action of β-glucosidase in soybeans during soymilk manufacturing process resulted in an increase in the objectionable after-taste. It was, therefore, considered that the

conversion of malonyl isoflavone glycosides into isoflavone glycosides during processing was necessary to produce soymilk with a very low degree of objectionable after taste.

Experimental

Spectroscopy. UV spectra (in methanol) were measured by a JASCO U-30 spectrometer, and NMR spectra were recorded on a JEOL GSX-400 spectrometer (¹H-NMR at 400 MHz, and ¹³C-NMR at 100 MHz in DMSO-*d*₆, with SiMe₄ as an internal standard). IR spectra (in KBr) were recorded with a JASCO A-202 instrument and mass spectra were recorded with a JEOL JMS HX-105.

Isolation of the isoflavone compounds. Soybean seed hypocotyls (500 g), which had been kindly supplied by Perikan Co., Ltd., were milled and extracted with a 10-fold volume of 70% aqueous ethanol at room temperature for 30 min. After filtering, the residue was repeatedly extracted three times. After the combined filtrate was evaporated to dryness under reduced pressure, the extract was partitioned between water and 1-butanol (1:1, v/v). After again evaporating to dryness, the butanol layer was repeatedly fractionated by Sephadex LH-20 column chromatography (5 × 74 cm, methanol). TLC analysis showed successive elution of the soybean saponin A group, soybean saponin B group, glycitin, daidzin, and genistin fractions. Glycitin, compound 5, daidzin and compound 4 were isolated from

the glycitin and daidzin fraction by HPLC (YMC-Pack ODS AM-323-7 column, 10 × 250 mm; mobile phase of 45% methanol). Genistin and compound 6 were obtained from the genistin fraction by HPLC (YMC-Pack ODS AM-323-7 column, 10 × 250 mm; mobile phase of 50% methanol). 6''-O-Acetyldaidzin, 6''-O-acetylglucitin and 6''-O-acetylgenistin were isolated according to procedure reported in the previous paper.¹¹⁾

Quantitative analyses of the isoflavones. Whole mature soybean seeds were lyophilized in a freeze dryer (FD-550, Tokyo Rikakikai Co., Ltd.) and divided into the seed coat, cotyledon and hypocotyl (containing the plumule and radicle) parts. After each part had been milled, 0.5 g of the milled sample was extracted in screw-capped test tubes with 10 volumes of 70% ethanol for 24 hr at room temperature or for 15 hr at 80 °C. After centrifugation, the supernatant was used directly in quantitative HPLC analyses.

To examine the change in isoflavone content during the development of the beans, the Maple Arrow variety was used. Plants were grown from seed under controlled conditions in a phytotron. The flowers were tagged every day, and the pods were harvested at various stages of maturation between 25 and 60 days after flowering. The immature seeds in the pods were lyophilized, milled and extracted with 70% aqueous ethanol at room temperature. After centrifugation, the supernatant was used directly in quantitative HPLC analyses.

TLC and HPLC analyses. TLC was carried out on a Kieselgel 60 F₂₅₄ plate (Merck), using chloroform-methanol-2% acetic acid (7:3:1, v/v lower layer). The components on TLC plate were visualized by heating a 120 °C for 10 min after spraying with 10% H₂SO₄. R_f values of compounds 1-9 were 0.41, 0.45, 0.43, 0.08, 0.12, 0.10, 0.56, 0.61 and 0.59, respectively.

Quantitative HPLC analyses were performed on a YMC-pack ODS-AM-303 column (250 × 4.6 mm), using a linear gradient of acetonitrile from 15 to 35% containing constant 0.1% acetic acid in 50 min. The solvent flow rate was 1 ml/min and the absorption was measured at 260 nm. The instrument used was a Waters HPLC system, comprising a Model 600E multi-solvent delivery system, Model 484 UV detector and Model 741 data module. Standard solutions used for quantitative analysis were prepared from 9 kinds of isoflavone glycosides isolated by HPLC and 3 kinds of aglycones obtained from the corresponding isoflavone glycosides by 5% H₂SO₄ hydrolysis in 50% ethanol.

Threshold value. Each sample solution (10⁻¹ mM) was diluted stepwise in tenths to a final concentration of 10⁻⁷ mM. The tests were carried out initially with the 10⁻⁷ mM sample solution until the panelists detected the taste of the sample. This lowest detectable concentration is defined as the threshold value.

Compound 4. mp 169 °C. UV λ_{\max} (nm) MeOH: 231 sh, 248 sh, 258, 303 (log ϵ 4.37, 4.47, 4.50, 4.00); + MeONa: 237 sh, 245 sh, 279, 300 sh; + AlCl₃: 231 sh, 247 sh, 258, 303 sh; + AlCl₃-HCl: 231 sh, 247 sh, 258, 303 sh; + NaOAc: 248 sh, 259, 304 sh. IR ν_{\max} (KBr): 1735, 1695 cm⁻¹. High-resolution FAB-MS: found, m/z 503.1181 [M+H]⁺; calcd. for C₂₄H₂₃O₁₂, 503.1188. FAB-MS: m/z 503 [M+H]⁺, 417 [M+H-86]⁺, 255 [M+H-86-162]⁺. EI-MS: m/z 254 [M-86-162]⁺, 137, 118.

Compound 5. mp 145 °C. UV λ_{\max} (nm) NeOH: 227 sh, 260, 318 (log ϵ 4.25, 4.42, 3.95); + MeONa: 284, 322 sh; + AlCl₃: 227 sh, 260, 317; + AlCl₃-HCl: 227 sh, 260, 318; + NaOAc: 260.317. IR ν_{\max} (KBr): 1735, 1695 cm⁻¹. High-resolution FAB-MS: found, m/z 533.1285 [M+H]⁺; calcd. for C₂₅H₂₃O₁₃, 533.1293. FAB-MS: m/z 533 [M+H]⁺, 447 [M+H-86]⁺, 285 [M+H-86-162]⁺. EI-MS: m/z 284 [M-86-162]⁺, 166, 118.

Compound 6. mp 162 °C. UV λ_{\max} (nm) MeOH: 260, 323 sh (log ϵ 4.51, 3.59); + MeONa: 271, 322 sh, 357 sh; + AlCl₃: 272, 308 sh, 380; + AlCl₃-HCl: 272, 308 sh, 379; + NaOAc: 261.329 sh. IR ν_{\max} (KBr): 1735, 1695 cm⁻¹. High-resolution FAB-MS: found, m/z 519.1169 [M+H]⁺; calcd. for C₂₄H₂₃O₁₃, 519.1137. FAB-MS: m/z 519 [M+H]⁺, 433 [M+H-86]⁺, 271 [M+H-86-162]⁺.

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λ_{\max} (nm) MeOH: 231 sh.
 17, 4.50, 4.00; + MeONa:
 231 sh, 247 sh, 258.
 AlCl_3 : 231 sh, 247 sh, 258.
 7 sh, 258, 303 sh; + NaOAc:
 (KBr): 1735, 1695 cm^{-1} .
 d, m/z : 503.1181 $[\text{M} + \text{H}]^+$.
 188. FAB-MS: m/z 503
 255 $[\text{M} + \text{H} - 86 - 162]^+$.
 $^+$, 137, 118.

λ_{\max} (nm) NeOH: 227 sh.
 20; + MeONa: 284, 322 sh;
 AlCl_3 -HCl: 227 sh, 260, 318;
 (KBr): 1735, 1695 cm^{-1} .
 d, m/z : 533.1285 $[\text{M} + \text{H}]^+$.
 203. FAB-MS: m/z 533 $[\text{M}$
 285 $[\text{M} + \text{H} - 86 - 162]^+$.
 $^+$, 166, 118.

V λ_{\max} (nm) MeOH: 260.
 20Na: 271, 322 sh, 357 sh;
 AlCl_3 -HCl: 272, 308 sh, 379;
 (KBr): 1735, 1695 cm^{-1} .
 d, m/z : 519.1169 $[\text{M} + \text{H}]^+$.
 1137. FAB-MS: m/z 519
 271 $[\text{M} + \text{H} - 86 - 162]^+$.

ank Mr. Takayanagi and
 University in the Faculty of
 cements of NMR and mass
 2000 of the AFRC Institute
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ISOLATION OF ISOFLAVONOIDS POSSESSING ANTIOXIDANT ACTIVITY FROM THE FERMENTATION BROTH OF *STREPTOMYCES* SP.

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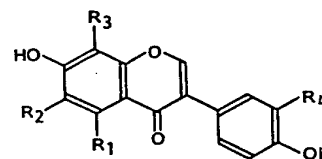
(Received for publication April 17, 1989)

Three antioxidant isoflavonoids characterized as 4',7,8-trihydroxyisoflavone (1), 3',4',7-trihydroxyisoflavone (2) and 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3) were isolated from the cultured broth of *Streptomyces* sp. OH-1049. Among them, 3 is a novel isoflavonoid possessing a chlorine atom in the molecule.

In *in vitro* studies, these antibiotics were found to possess antioxidant activity whereas showed almost no cytotoxic activities against HeLa S₃ cells.

In the course of a screening program for novel antibiotics showing antioxidant activity, a fraction of fermentation broth of *Streptomyces* sp. OH-1049 which had been isolated from a soil sample collected in Kanagawa Prefecture, Japan showed potent antioxidant activity and three active components, 4',7,8-trihydroxyisoflavone (1), 3',4',7-trihydroxyisoflavone (2) and 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3), were isolated.

The present paper deals with the taxonomy of the producing strain together with the production, isolation and biological properties of these antibiotics. Chemical characterization and structural studies of these compounds will be reported in a separate paper¹⁾.



- 1 R₁=R₂=R₄=H R₃=OH
- 2 R₁=R₂=R₃=H R₄=OH
- 3 R₁=R₄=OH R₂=H R₃=Cl
- 4 R₁=R₃=R₄=H R₂=OH
- 5 R₁=R₂=R₃=R₄=H

Materials and Methods

Taxonomic Studies

The type of diaminopimelic acid (DAP) in the microorganisms was determined by the method of HASEGAWA *et al.*²⁾

To investigate the cultural and physiological characteristics, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB³⁾ and those recommended by WAKSMAN⁴⁾ were used. Cultures were observed after incubation at 27°C for 2 weeks. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th Ed.)⁵⁾. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon source at 27°C.

Antioxidant Activity Tests

Male Wistar rats were sacrificed by decapitation and exsanguination. To prepare a microsomal fraction, livers were collected and homogenized with cold 1.15% KCl and centrifuged at $10,000 \times g$ for 30 minutes. The microsomal fraction was sedimented by ultracentrifugation at $78,000 \times g$ for 1 hour. The microsomal fraction was diluted with 150 mM KCl - 50 mM Tris-HCl buffer, pH 7.5 to make the concentration of protein to be 1.7 mg/ml. To this microsomal solution (1.0 ml) was added 0.0425 M NADPH (0.1 ml), 2.0 mg/ml doxorubicin (ADM) and a sample solution (0.1 ml) and the total volume was adjusted to be 1.7 ml with KCl-Tris buffer (pH 7.4) and incubated for 1 hour at 37°C.

After the incubation, lipid peroxide level was determined by the method of UCHIYAMA and MIHARA⁴² slightly modified as follows. The solution (0.5 ml) was taken from each test tube and was mixed with 1% phosphoric acid (3.0 ml) and 0.67% thiobarbital (TBA) solution (1.0 ml). The mixture was heated on a boiling water for 45 minutes. After cooling, 4.0 ml of BuOH was added and mixed vigorously. The butanol phase was separated by centrifugation and absorbance was measured at 520 and 535 nm. The difference was used as the TBA value. As a standard solution, 10 nmol of 1,1,3,3-tetraethoxypropane was used.

4',6,7-Trihydroxyisoflavone (4) and daidzein (5) were purchased from Funakoshi Pharmaceutical Co., Ltd., Tokyo, Japan.

Antimicrobial Activity Test

The antimicrobial activities of 1, 2 and 3 were tested using 6 mm paper discs (Toyo Seisakusho Co., Ltd.) and Mueller-Hinton agar medium (Difco) for bacteria and potato broth agar medium for fungi or yeasts. Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Anti HeLa S₃ Activity Tests

HeLa S₃ cells were maintained in monolayers in EAGLE's minimum essential medium (MEM) supplemented with 10% bovine serum and an antibiotic (60 µg/ml of kanamycin) at 37°C.

To determine the cytotoxicity of 1, 2 and 3, HeLa S₃ cells (5×10^4) in 2 ml of medium were placed in a 30-mm Petri dish and incubated for 48 hours at 37°C in a 5% CO₂ - 95% air atmosphere. Each culture dish was filled with fresh medium containing a different concentration of the antibiotic. After further 72 hours incubation, the HeLa S₃ cells were counted in hemocytometer.

Results

Taxonomy of the Producing Strain OH-1049

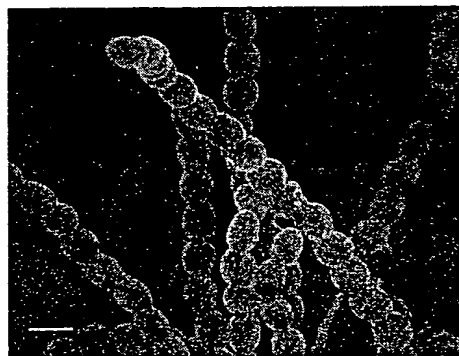
The vegetative mycelia grow abundantly on both synthetic and complex agar media, and do not show fragmentation into coccoid or bacillary elements. The velvety aerial mycelia grow abundantly on inorganic salts - starch agar and glycerol - asparagine agar. The mature sporophores were of the *Rectiflexibiles* type and had more than 20 spores per chain. The spores were oval in shape, $1.1 \times 0.7 \mu\text{m}$ in size and had a smooth surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

The type of DAP in the cell wall was determined as LL by the method of HASEGAWA *et al.*²³.

The cultural and the utilization of carbon sources of OH-1049 are shown in Tables 1, 2 and 3, respectively.

Fig. 1. Scanning electron micrograph of spore chains of strain OH-1049 grown on inorganic salts - starch agar for 14 days.

Bar represents 1.0 µm.



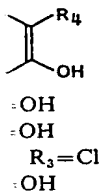
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Table 1. Cultural characteristics of strain OH-1049.

Medium	Cultural characteristics
Yeast extract - malt extract agar ^a	G: Moderate, light ivory (2ca) R: Light mustard tan (2ie) AM: Moderate, velvety, ashes (5fe) SP: None
Oatmeal agar ^a	G: Moderate, penetrant, light ivory (2ca) R: Light mustard tan (2ie) AM: Moderate, velvety shadow gray (5ih) SP: None
Inorganic salts - starch agar ^a	G: Good, light wheat (2ea) R: Light mustard tan (2ie) AM: Abundant, velvety, ashes (5fe) SP: None
Glycerol - asparagine agar	G: Good, light ivory (2ca) R: Covert tan (2ge) AM: Abundant, velvety, ashes (5fe) SP: None
Glucose - asparagine agar	G: Good, light ivory (2ca) R: Light mustard tan (2ie) AM: Abundant, velvety, silver gray (3fe) SP: None
Peptone - yeast extract - iron agar ^a	G: Good, rose beige (4gc) R: Light amber (3ic) AM: Moderate, velvety, cream (1 1/2ca) SP: Maple (4le)
Tyrosine agar ^a	G: Good, ivory (2db) R: Clove brown (3pl) AM: Moderate, velvety, covert gray (2fe) SP: None
Sucrose - nitrate agar ^a	G: Poor, colorless R: Light ivory (2ca) AM: Poor, powdery, light beige (3ec) SP: None
Glucose - nitrate agar ^a	G: Poor, colorless R: Pearl (3ba) AM: Poor, sand (3cb) SP: None
Glycerol - calcium malate agar ^b	G: Good, light ivory (2ca) R: Sand (3cb) AM: Moderate, velvety, ashes (5fe) SP: None
Glucose - peptone agar ^b	G: Good, light ivory (2ca) R: Light wheat (2ea) AM: Moderate, velvety, white (a) or pearl gray (13dc) SP: None
Nutrient agar ^b	G: Good, light wheat (2ea) R: Bamboo (2gc) AM: Abundant, velvety, pussywillow gray (5dc) SP: None

Abbreviations: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

^a Medium recommended by ISP.

^b Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain OH-1049.

Melanin formation	—
Tyrosinase reaction	—
H ₂ S production	—
Liquefaction of gelatin (21~22°C)	±
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	—
Cellulolytic activity	—
Hydrolysis of starch	+
Temperature range for growth	10~37°C

+ : Active, ± : weakly active, — : inactive.

The strain exhibits the following properties. Sporophore, *Rectiflexibiles*; spores, oval and smooth surface; color of vegetative mycelia, light ivory; color of aerial mycelia, gray; soluble pigment, maple; DAP isomer in cell wall, LL-type.

Based on the taxonomic properties described above, strain OH-1049 is considered to belong to the genus *Streptomyces* and to be a strain of the gray series of the PRIDHAM and TRESNER's system²⁾. The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. OH-1049 and the accession No. is FERM P-9858.

Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 80 ml seed medium consisting of starch 1.5%, glucose 0.2%, peptone 0.25%, yeast extract 0.15%, meat extract 0.3% and CaCO₃ 0.25% (pH 7.0 before sterilization). The flasks were incubated at 27°C for 72 hours on a reciprocal shaker. Then 240 ml of the resulting culture were transferred to a 30-liter fermenter containing 20 liters of medium consisting of glycerol 2.0%, soybean meal 2.0% and NaCl 0.3% (pH 7.0 before sterilization). The fermentation was carried out at 27°C for 72 hours using an agitation rate of 160 rpm and an aeration rate of 60 liters/minute. The 20 liters of the resulting culture were transferred to a 400-liter fermenter containing 200 liters of the same medium described above, and fermentation was carried out at the same conditions described above.

The whole broth of *Streptomyces* sp. OH-1049 (200 liters) was extracted with EtOAc (200 liters) and the EtOAc layer was concentrated *in vacuo* to about 10 liters, washed with H₂O (5 liters) and dried over Na₂SO₄ (anhydrous). Concentration of the EtOAc layer resulted in a brown oil.

The brown oil was chromatographed over Silica gel 60 (Merck) using CHCl₃ - MeOH as solvent. Fractions exhibiting antioxidant activity were collected and rechromatography of the active fractions over Sephadex LH-20 column chromatography using MeOH as solvent gave crude mixture of active components. Finally, three active components were purified through the preparative TLC using CHCl₃ - MeOH (9 : 1) as solvent and/or preparative HPLC using a column of YMC A-303 (Yamamura Chemical Laboratory; 4.6 i.d. × 250 mm) eluted with MeOH - H₂O (39 : 11) as solvent.

Table 3. Utilization of carbon sources by strain OH-1049.

Utilized:	D-Glucose, D-fructose, D-mannitol, L-arabinose, D-xylose
Weakly utilized:	Sucrose
Not utilized:	L-Rhamnose, <i>D</i> -inositol, raffinose, melibiose

Table 4. Antioxidant activity of 1~5 and α -tocopherol.

Sample	Inhibitory percent of malondialdehyde generation			
	20 ^a	4	0.8	0.16
1	100	100	100	40
2	100	100	70	41
3	100	100	80	32
4	100	100	90	70
5	77	20	6	6
α -Tocopherol	100	100	31	34

^a Concentration of sample (μ g/ml).

Antimicrobial Activity Test

Isoflavonoids 1~3 showed no antimicrobial activities at the concentration of 100 $\mu\text{g/ml}$ against *Xanthomonas oryzae* KB 88, *Candida albicans* KF 1, *Saccharomyces sake* KF 26, *Mucor racemosus* KF 223 (IFO 4581), *Piricularia oryzae* KF 180, *Aspergillus niger* KF 103 (ATCC 6275), *Staphylococcus aureus* KB 34 (FDA 209P), *Bacillus subtilis* KB 27 (PCI 219), *Escherichia coli* KB 8 (NIHJ), *E. coli* KB 176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB 105 (P3), *Micrococcus luteus* KB 40 (PCI 1001), *Bacteroides fragilis* KB 169, *Mycobacterium smegmatis* KB 42 (ATCC 607) and *Acholeplasma laidlawii* PG 8 KB 174.

Antioxidant Activity Test

Antioxidant activities of 1~5 and α -tocopherol are shown in Table 4. The antioxidant activities of isoflavonoids 1~3 were comparable to those of 4 and α -tocopherol.

Anti HeLa S₃ Activity Test

Cytocidal activities (MIC) of compounds 1~5 against HeLa S₃ cells were 12.5, 6.3, 25.0, 12.5 and 3.2 $\mu\text{g/ml}$, respectively.

Discussion

A novel antibiotic, 8-chloro-3',4',5,7-tetrahydroxyisoflavone (3) was isolated from the cultured broth of *Streptomyces* sp. OH-1049 together with 4',7,8-trihydroxyisoflavone (1) and 3',4',7-trihydroxyisoflavone (2).

Compounds 1~3 showed comparable antioxidant activity to those of α -tocopherol and 4',6,7-trihydroxyisoflavone (4) whereas daidzein (5) showed only weak activity (Table 4). An isoflavone derivative, 4',6,7-trihydroxyisoflavone (4) was isolated as an antioxidant component of Indonesian food "Tempeh" which was prepared by the action of *Rhizopus oryzae* on boiled soybeans^{6,9} and daidzein (5) was obtained as an aglycone of daidzin (daidzein-7-O-glucoside) isolated from soybeans¹⁰.

Compounds 1~3 were only obtained from the fermentation broth of *Streptomyces* sp. OH-1049 using a cultivation medium containing soybean meal, whereas these compounds could not be obtained from the untreated soybean meal. These facts indicated that both soybean meal and *Streptomyces* sp. OH-1049 are necessary for the production of 1~3. The physico-chemical properties and structure elucidation procedures of compounds 1~3 are described in a subsequent paper¹¹.

We are now investigating the biological activities of these compounds and their related compounds further and results will be reported elsewhere.

Acknowledgment

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of 100 µg/ml against
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EFFECTS OF INHIBITORS OF PROTEIN TYROSINE KINASE ACTIVITY AND/OR PHOSPHATIDYLINOSITOL TURNOVER ON DIFFERENTIATION OF SOME HUMAN MYELOMONOCYTIC LEUKEMIA CELLS

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Abstract—The activities of protein tyrosine kinase and phosphatidylinositol turnover have been found to be associated with cell growth and differentiation. We examined the effects of some inhibitors for these biochemical activities in human myelogenous leukemia cells. Genistein, which is known to inhibit the activities of protein tyrosine kinase, phosphatidylinositol turnover and topoisomerase II, induced nitroblue tetrazolium (NBT) reduction and lysozyme activity in ML-1, HL-60 and U937 cells. Morphological studies showed that genistein-induced differentiation of myeloblastic ML-1 cells into promyelocytes and of promyelocytic HL-60 cells into mature granulocytes. The differentiation-inducing effect of genistein was augmented by addition of $1\alpha,25$ -dihydroxyvitamin D_3 (VD_3) or retinoic acid, VD_3 being more effective than retinoic acid. Methyl 2,5-dihydroxycinnamate, a protein tyrosine kinase inhibitor, had only a weak effect in inducing differentiation of ML-1 cells. On the other hand, psi-tectorigenin was more effective than genistein in inducing the differentiations of ML-1 and HL-60 cells. Psi-tectorigenin is reported to inhibit phosphatidylinositol turnover without inhibiting protein tyrosine kinase. Thus modulation of phosphatidylinositol turnover might be more important than that of protein tyrosine kinase activity for differentiation of some myelogenous leukemia cells.

Key words: Differentiation, phosphatidylinositol turnover, psi-tectorigenin, myelomonocytic leukemia cells.

INTRODUCTION

PHOSPHATIDYLINOSITOL turnover is implicated in cellular proliferation and differentiation [1, 2]. Activation of this system increases levels of diacylglycerol, which is an activator of protein kinase C, and inositol triphosphates, which rise intracellular Ca^{2+} level [3, 4]. These second messenger molecules may play an important role in cellular signal transduction. On the other hand, protein tyrosine kinase activity is involved in the control of cell proliferation, carcinogenesis, and cell differentiation [5, 6]. Inhibitors of protein tyrosine kinase or phosphatidylinositol turnover are useful for studying

these properties and functions. Genistein, which is known to be an inhibitor of protein tyrosine kinase activity, was found to induce differentiation of human myelogenous leukemia HL-60 cells [7] and a mouse erythroleukemia cell line [8]. However, this compound has effects on several other cellular events, such as DNA strand breakage [9] and phosphatidylinositol turnover [10] as well as tyrosine phosphorylation [11].

Thus it may influence cell differentiation through several different mechanisms. Therefore, in this study we investigated the effects of genistein and functionally related compounds on growth and differentiation of some human myelogenous leukemia cells. Our results suggested that induction of differentiation by genistein might be associated with its effects on phosphatidylinositol turnover in some human myelogenous leukemia cells.

Abbreviations: NBT, nitroblue tetrazolium; VD_3 , $1\alpha,25$ -dihydroxyvitamin D_3 .

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MATERIALS AND METHODS

Cell lines and cell culture

Human leukemia ML-1 [12], HL-60 [13] and U937 cells [14] were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ in air.

Assay of cell growth and properties of differentiated cells

Cells (10⁵/ml) were suspended in 2 ml of culture medium and cultured with or without inducers in multidishes (Costar, Cambridge, MA). Cell numbers were counted with a Model ZM Coulter Counter (Coulter Electronics, Luton, England) after various treatments for 5 days. Cellular viabilities after all the treatments in this study were more than 80%, determined by exclusion of trypan blue. Nitroblue tetrazolium (NBT) reduction was assayed colorimetrically as described previously [15]. The reaction was stopped by adding 5 M HCl (1 M final concentration). The suspension was stood for 1 h at room temperature and then centrifuged and the medium was discarded. The formazan deposits were solubilized by adding dimethyl sulfoxide, and the absorption of the formazan solution at 560 nm was measured in a spectrometer. Lysozyme activity was determined using lysoplates containing 1% agar, 1/15 M sodium phosphate buffer (pH 6.6), 50 mM NaCl, and heat-killed *Micrococcus lysodeikticus* (0.5 mg/ml) [16]. The percentage of cells that were morphologically similar to mature granulocytes or macrophages was determined in smears treated with May-Grünwald-Giemsa stain.

Materials

Genistein was purchased from Funakoshi Pharmaceutical Co., Tokyo; a stock solution was prepared in dimethyl sulfoxide and diluted with ethanol before use. Methyl 2,5-dihydroxycinnamate and psi-tectorigenin were prepared as reported previously [10,17]. 1 α ,25-dihydroxyvitamin D₃ (VD₃) was obtained from Chugai Pharmaceutical Co., Tokyo. Retinoic acid (all-trans) was purchased from Sigma Chemical Co., St Louis.

RESULTS

Effects of genistein on growth and differentiation of leukemia cell lines

Genistein, an isoflavone compound isolated from the fermentation broth of *Pseudomonas* sp., inhibits protein tyrosine kinase [11] and topoisomerase activities [9], and phosphatidylinositol turnover [10]. We examined the effect of this compound on the growth and differentiation of various human myelomonocytic leukemia cell lines. On incubation with genistein for 5 days, the growth of ML-1 cells was inhibited dose-dependently, 2.7 μ g/ml of genistein causing 50% inhibition. NBT reduction is a typical marker of myelomonocytic differentiation of the cells [19]. The NBT reduction activity of untreated ML-1 cells (0.79 A₅₆₀/10⁷ cells) was increased dose-dependently by genistein (Fig. 1a). Lysozyme activity, another marker of functional differentiation [16], was increased about 6-fold by culture of ML-1 cells with

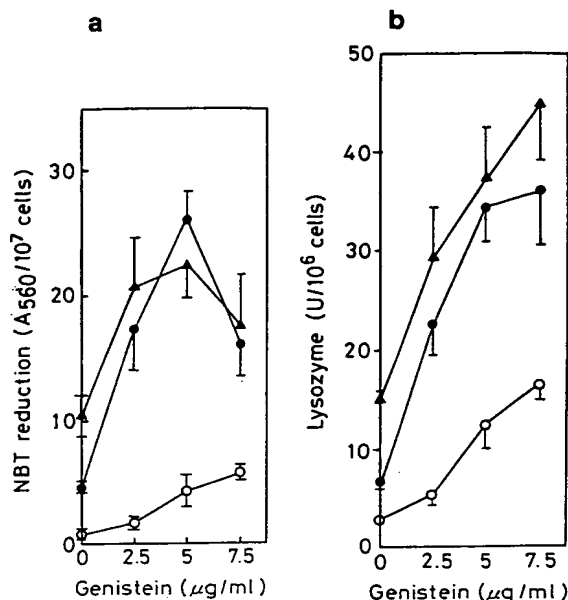


FIG. 1. Induction of NBT reduction (a) and lysozyme activity (b) of ML-1 cells by treatment with genistein in the presence and absence of VD₃. Cells were treated with various concentrations of genistein in combination with 0 ng/ml (○), 1 ng/ml (●) or 10 ng/ml (▲) of VD₃ for 5 days. Values are averages \pm S.D. from three separate experiments.

7.5 μ g/ml genistein (Fig. 1b). Examination of cell smears showed that untreated ML-1 cells had large round nuclei and basophilic cytoplasm like myeloblasts, but that many genistein-treated cells had abundant azurophilic granules like promyelocytes (Fig. 2). Treatment of the cells with a higher concentration of genistein (10 μ g/ml) for more than 6 days did not induce their morphological differentiation into more mature granulocytes (Table 1).

VD₃ has been shown to induce monocytic differentiation of several leukemia cell lines, including ML-1 cells [19]. The NBT reducing activities of cells treated with 1 and 10 ng/ml VD₃ were 4.45 and 10.34 A₅₆₀/10⁷ cells, respectively. The effect of VD₃ was augmented in the presence of genistein (Fig. 1). After treatment of the cells with 2.5 and 5 μ g/ml genistein in the presence of 1 ng/ml VD₃, their NBT reducing activities were 17.27 and 25.99 A₅₆₀/10⁷ cells, respectively. Similar results were obtained on treatment with genistein in the presence of 10 ng/ml VD₃. Figure 1b shows that, at suboptimal concentrations, genistein and VD₃ had additive, or more than additive effects in induction of lysozyme activity of the cells. Morphological study showed that genistein-treated ML-1 cells were promyelocytic, but that VD₃ induced monocytic differentiation of ML-1 cells (Table 1). After treatment with 5 μ g/ml genistein and 1 ng/

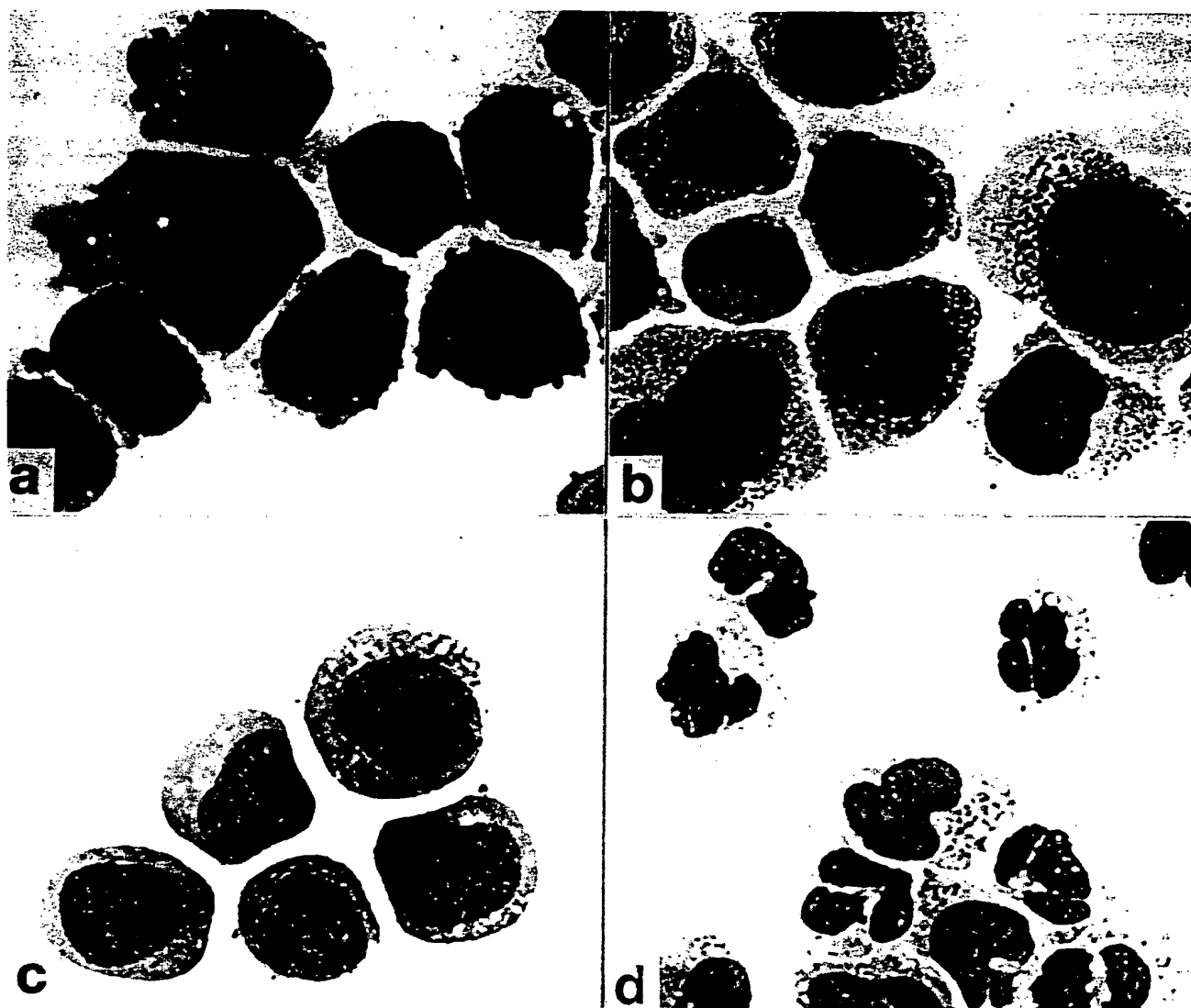


FIG. 2. Morphological changes of ML-1 and HL-60 cells induced by genistein. Cells were treated with 4 $\mu\text{g}/\text{ml}$ of genistein for 6 days: (a) untreated ML-1 cells; (b) genistein-treated ML-1 cells; (c) untreated HL-60 cells; (d) genistein-treated HL-60 cells.

TABLE 1. EFFECTS OF GENISTEIN AND PSI-TECTORIGENIN ON DIFFERENTIATION OF ML-1 CELLS WITH AND WITHOUT VD₃ OR RETINOIC ACID

Treatment	Induction of differentiation						
	NBT reduction (A ₅₆₀ /10 ⁷ cells)	Lysozyme (U/10 ⁶ cells)	Morphological changes (%)				
			Bla	Pro	MyeMo	Gra	Mø
None	0.79	2.9	89	10	1	0	0
Genistein	4.34	12.3	14	74	11	0	1
VD ₃	4.45	6.7	32	41	26	0	1
VD ₃ +genistein	25.99	34.5	0	15	64	0	21
RA	2.45	4.0	23	30	38	9	0
RA+genistein	8.55	9.4	1	17	71	10	1
Psi-tectorigenin	8.09	—	14	42	41	0	3

ML-1 cells were cultured with or without 5 µg/ml of genistein in the presence or absence of 1 ng/ml of VD₃ or 4 × 10⁻⁹ M retinoic acid (RA) for 5 days. The cells were also treated with 10 µg/ml of psi-tectorigenin for 5 days. Bla, blasts; Pro, promyelocytes; MyeMo, myelocytes and monocytes; Gra, metamyelocytes and mature granulocytes; Mø, macrophages.

ml VD₃, 21% of the cells were macrophage-like, whereas after treatment with 1 ng/ml VD₃ alone, only 1% were macrophage-like.

Retinoic acid has been shown to be a potent inducer of granulocytic differentiation of several human leukemia cell lines including ML-1 cells [19]. We examined its effect in combination with genistein on differentiation of ML-1 cells. Exposure of the cells to 4 × 10⁻⁹ M retinoic acid induced about 3-fold increase in their activity for reducing NBT. Genistein enhanced the effect of 4 × 10⁻⁹ M retinoic acid additively (Table 1). Similar results were obtained in studies on morphological changes of the cells treated with genistein in the presence of retinoic acid.

Next, we examined the effect of genistein on the other human myelomonocytic leukemia cell lines. Genistein inhibited the growth of monoblastic leukemia U937 cells, causing 50% inhibition at 2.2 µg/ml, and induced NBT reduction dose-dependently. VD₃ and retinoic acid are effective inducers of differentiation of U937 cells. On treatment with suboptimal concentrations of VD₃ or retinoic acid, NBT reduction was augmented additively or more than additively by 5 µg/ml genistein (Table 2). Growth of promyelocytic leukemia HL-60 cells was less sensitive to genistein, because 50% inhibition of growth was observed with genistein at 6.7 µg/ml. Genistein alone, however, induced morphological differentiation of HL-60 cells into mature granulocytes (Fig. 2). NBT reduction was also enhanced additively or more than additively by genistein in cells treated with retinoic acid or VD₃ (Table 2).

TABLE 2. INDUCTION OF NBT REDUCTION IN SOME MYELOMONOCYTIC LEUKEMIA CELLS BY GENISTEIN IN THE PRESENCE OF VD₃ OR RETINOIC ACID

Treatment	NBT reduction (A ₅₆₀ /10 ⁷ cells)					
	ML-1 cells		HL-60 cells		U937 cells	
	-G	+G	-G	+G	-G	+G
None	0.79	4.34	1.00	7.23	0.55	2.47
VD ₃ , 1 ng/ml	4.45	25.99	1.81	12.52	1.95	5.84
VD ₃ , 10 ng/ml	10.33	22.42	5.19	14.14	—	—
RA, 4 × 10 ⁻⁹ M	2.45	8.55	1.06	11.04	1.76	5.54

Cells were cultured with or without 5 µg/ml of genistein (G) for 5 days. RA, retinoic acid.

Effects of methyl 2,5-dihydroxycinnamate on growth and differentiation

To determine whether the differentiation-inducing effect of genistein on leukemia cells was due to inhibition of protein tyrosine kinase or some other mechanism(s), we examined the effect of another protein tyrosine kinase inhibitor, methyl 2,5-dihydroxycinnamate [17], on ML-1 cells. This compound inhibited the growth of ML-1 cells, causing 50% inhibition at 0.54 µg/ml when incubated with the cells for 5 days. It did not significantly increase NBT reduction or lysozyme activity of the cells. It also caused only slight enhancement of NBT reduction and induction of lysozyme activity in VD₃- or retinoic acid-treated ML-1 cells (Fig. 3). Even at high concentration, it did not induce functional or mor-

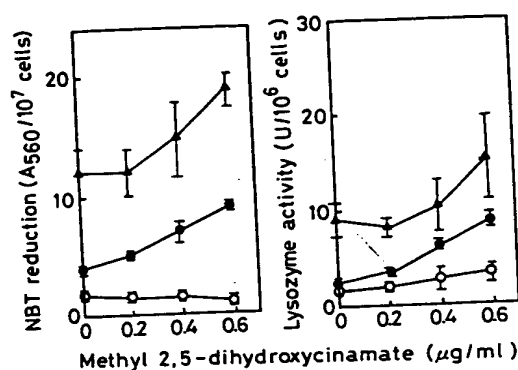


FIG. 3. Effects of methyl 2,5-dihydroxycinnamate on induction of NBT reduction and lysozyme activity of ML-1 cells. Cells were treated with various concentrations of methyl 2,5-dihydroxycinnamate without (○), or with 4×10^{-8} M retinoic acid (●) or 10 ng/ml of VD_3 (▲) for 5 days.

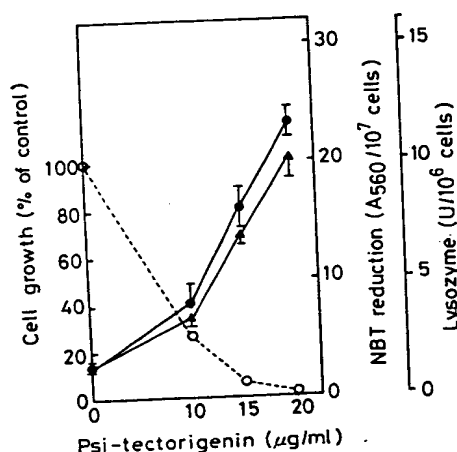


FIG. 4. Effects of psi-tectorigenin on growth and differentiation of ML-1 cells. Cells were treated with various concentrations of psi-tectorigenin for 5 days. ○—○, cell growth; ●—●, NBT reduction; ▲—▲, lysozyme activity.

phological differentiation of HL-60 or U937 cells (data not shown).

Effects of psi-tectorigenin on growth and differentiation of leukemia cells

Psi-tectorigenin, isolated from *Aspergillus* sp., inhibits phosphatidylinositol turnover more effectively than genistein [10]. Psi-tectorigenin inhibited the growth of ML-1 cells, causing 50% inhibition of growth at 6.4 μ g/ml. It also induced dose-dependent NBT reduction and lysozyme activity of ML-1 cells (Fig. 4). It caused morphological changes of ML-1 cells (Table 1), and its differentiation-inducing activity was greater than that of genistein. Psi-tectorigenin less than 5 μ g/ml did not induce NBT reducing activity of ML-1 cells but significantly enhanced that by suboptimal dose of VD_3 (1 ng/ml) (Fig. 5). On

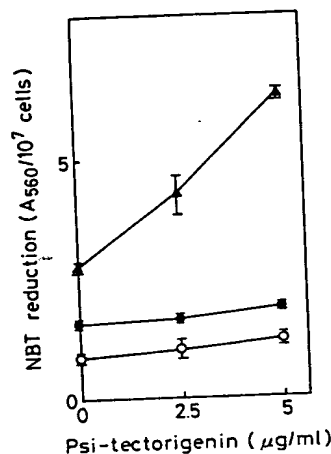


FIG. 5. Effects of VD_3 and retinoic acid on induction of NBT reduction of ML-1 cells by psi-tectorigenin. Cells (2×10^5 /ml) were treated with various concentrations of psi-tectorigenin without (○), or with 4×10^{-9} M retinoic acid (●) or 1 ng/ml of VD_3 (▲) for 5 days.

the contrary, the combined effects of psi-tectorigenin and retinoic acid seemed just additive. Psi-tectorigenin induced significant differentiation of HL-60 cells, but U937 cells were less sensitive to its effects in inhibiting growth and inducing NBT reduction and lysozyme activity (data not shown).

DISCUSSION

In the present investigation we found that genistein induced differentiation of some human myelomonocytic leukemia cell lines. Genistein is reported to inhibit tyrosine phosphorylation [11], phosphatidylinositol turnover [10] and topoisomerase II activity [9]. Constantinou *et al.* recently reported that genistein induced monocytic differentiation of a variant clone of the HL-60 cell line, and suggested that the differentiation-inducing effect of genistein might be associated with inhibition of topoisomerase II activity [7]. However, genistein induced intracellular DNA damage at a concentration of more than 30 μ g/ml of genistein, whereas induction of differentiation was observed in cells treated with 5 μ g/ml of genistein. For more precise understanding of the differentiation-inducing effect of genistein, we examined the differentiation-inducing effects of two related compounds, methyl 2,5-dihydroxycinnamate [17], a protein tyrosine kinase inhibitor and psi-tectorigenin [10], a potent inhibitor of phosphatidylinositol turnover.

The present study showed that methyl 2,5-dihydroxycinnamate had only a slight effect on differentiation of leukemia cells, and previous results showed that herbimycin A was unable to induce

differentiation of HL-60, U937 and ML-1 cells, although this compound induced erythroid differentiation of K562 cells, which have p210^{bcr-abl} fusion protein with abnormally high protein tyrosine kinase activity [20]. Genistein inhibits protein tyrosine kinase competitively with ATP [11], while methyl 2,5-dihydroxycinnamate inhibits the enzyme competitively with peptide [21]. Herbimycin A reacts with the SH-groups of protein tyrosine kinase [22], resulting in inhibition of the enzyme activity. These results suggest that the differentiation-inducing activity of genistein on ML-1, HL-60 and U937 cells is unlikely to be associated with inhibition of protein tyrosine kinase activity.

On the other hand, psi-tectorigenin was effective in inducing differentiation of these leukemia cells. We have reported that this compound inhibited phosphatidylinositol turnover without inhibiting protein tyrosine kinase in cultured A431 cells [10]. Addition of psi-tectorigenin at 5–50 µg/ml inhibited formation of phosphatidic acid about 90% and that of phosphatidylinositol 90–99%, but at 50 µg/ml it did not inhibit protein tyrosine kinase activity of epidermal growth factor receptor *in situ*. The leukemia cells were induced to differentiate by 5–20 µg/ml of psi-tectorigenin. Furthermore, psi-tectorigenin was more potent than genistein in inducing NBT reduction of ML-1 cells (maximum inductions, 23.2 and 5.8 A₅₆₀/10⁷ cells). Previous reports indicated that phosphatidylinositol turnovers of murine and human leukemic cells decreased during induction of differentiation [2, 23, 24]. These findings suggest that phosphatidylinositol turnover may influence the differentiations of some myelomonocytic leukemia cells, although other possibilities cannot be ruled out.

Protein tyrosine kinase is reported to affect phosphatidylinositol turnover [1, 25–28]: protein tyrosine kinase is postulated to phosphorylate phosphatidylinositol kinase [26, 27] and phospholipase C [28], and then activate phosphatidylinositol turnover. Inhibition of protein tyrosine kinase might suppress phosphatidylinositol turnover and protein kinase C indirectly. On the other hand, phospholipase C and phorbol esters, activators of protein kinase C, have been reported to induce monocytic differentiation of HL-60 cells [29]. The present results indicated that HL-60 cells were induced to differentiate into mature granulocytes, but not into monocytes and macrophages, by genistein or psi-tectorigenin. Modulation of these signal transduction pathways may influence differentiation processes.

Myeloblastic ML-1 cells were induced to differentiate into promyelocytes, but not into more mature granulocytes by genistein even at high concentration. However, promyelocytic HL-60 cells

were induced to differentiate into mature granulocytes by this compound. These experimental systems may be useful for analyses of the molecular mechanisms involved in intermediate stages of granulocytic differentiation.

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13

COMMENTARY

EFFECTS OF FLAVONOIDS ON IMMUNE AND INFLAMMATORY CELL FUNCTIONS

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The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals such as the flavonoids. Some effects of the flavonoids on the function of T cells, B cells, NK cells, macrophages, mast cells, basophils, neutrophils, eosinophils and platelets will be described. Each of these cell types is involved in immunity and inflammation, and the effects of the flavonoids on these cells will be considered in this broad context. This commentary is selective and does not cover the literature exhaustively.

The flavonoids are comprised of a large group of low molecular weight polyphenolic secondary plant metabolites which possibly are important to the health and maintenance of herbivorous animals including humans. Some basic flavonoid structures, including many discussed herein, are shown in Fig. 1. Dietary exposure to flavonoids is not insignificant. For instance, the average Western diet contains approximately 1 g/day of mixed flavonoids [1]. Ingestible flavonoids are found in fruits, vegetables, nuts, seeds, stems, flowers, roots, bark, tea, wine and coffee. They are prominent components of citrus fruits and other food sources [2]. The flavonoids have long been considered to possess antiallergic and antiinflammatory activities. Their effects on a variety of inflammatory processes have been reviewed [3, 4]. They are also potent antioxidants and possess significant vitamin C-sparing activity [5, 6].

The flavonoids display a remarkable array of

biochemical and pharmacological actions [7-12], some of which suggest that certain members of this group of compounds significantly affect the function of the immune system. As will be described in greater detail, a number of flavonoids affect the function of enzyme systems critically involved in the immune response and the generation of inflammatory processes, namely, both tyrosine and serine-threonine protein kinases, phospholipase A₂, phospholipase C (PLC[†]), lipoxygenases and others. Recently, it has become evident that these enzymes are critically involved in signal transduction and cell activation processes involving cells of the immune system as well as other cells. Much of the information on flavonoid effects has been obtained with *in vitro* systems; more *in vivo* studies are very much warranted.

T lymphocytes

The convergence of immunologic research on the nature of T cell antigen recognition and investigations of signal transduction in T and B cells have led to new fundamental concepts. T cell proliferation follows the cooperative interaction of CD4, CD8 and the T cell receptor (TCR)-CD3 complex upon exposure to foreign antigens and in association with appropriate molecules of the major histocompatibility complex. It is now understood that the proliferative signal is generated by members of a family of protein tyrosine kinases (PTKs) which catalyze the phosphorylation of cellular substrates which in turn is accompanied by T cell proliferation [13]. Tyrosine phosphatases dephosphorylate the phosphoproteins returning the cell toward baseline conditions [14]. Certain flavonoids affect the activity of PTKs but nothing is known about their possible effect on tyrosine phosphatases.

T lymphocyte stimulation through the antigen receptor causes early activation of a tyrosine kinase [15-17] and the generation of phosphatidylinositol (PI) bisphosphate (PIP₂)-derived second messengers, namely inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), via activation of phospholipase C [18, 19]. Several cellular substrates are phosphorylated including TCR-zeta. The T cell PTK, p56^{lck}, may be involved in this process. Trevillyan *et al.* [17] showed that the isoflavone, genistein, a selective PTK inhibitor [20], blocked the activity of p56^{lck} in a concentration-dependent manner (IC₅₀ =

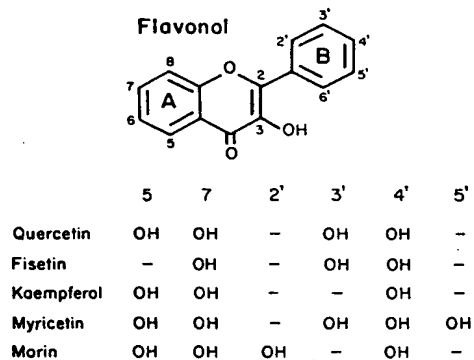
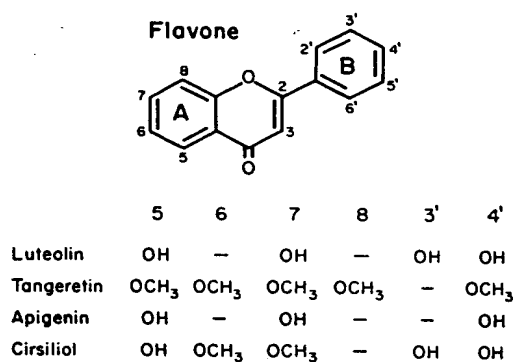
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† Abbreviations: PLC, phospholipase C; TCR, T cell receptor; PTK, protein tyrosine kinase; PI, phosphatidylinositol; PIP₂, phosphatidylinositol bisphosphate; IP₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; IL, interleukin; EGF, epidermal growth factor; PKC, protein kinase C; mAbs, monoclonal antibodies; TGP, tobacco glycoprotein; BSA, bovine serum albumin; SRBC, sheep red blood cells; PHA, phytohemagglutinin; DTH, delayed type hypersensitivity; DNFB, dinitrofluorobenzene; DMBA, dimethylbenzanthracene; TPA, tetradecanoyl phorbol acetate; EBV, Epstein-Barr virus; EA, early antigen; TNF, tumor necrosis factor; LT, leukotriene; PMA, phorbol 12-myristate 13-acetate; MPO, myeloperoxidase; and PAF, platelet-activating factor.

40 μ M). Inhibition of enzyme activity correlated with reduced interleukin-2 (IL-2) secretion and IL-2R expression but not with TCR-mediated PI hydrolysis.

Recently it has been demonstrated that CD45 tyrosine phosphatase is essential for coupling the T cell antigen receptor to the PI pathway [18]. Experiments of Ledbetter *et al.* [19] and others demonstrated that CD45 tyrosine phosphatase can serve as a regulator of TCR complex-mediated PLC activation in human peripheral blood lymphocytes. CD45 inhibited the increase in cytoplasmic Ca^{2+} concentration, suggesting that PI hydrolysis is regulated by CD45. Also, ligation of CD45 inhibited phosphorylation of tyrosine on specific substrates

during T cell activation. As well as an effect on PLC activation, CD45 may exert effects on cell activation through its enzymatic activity as a tyrosine phosphatase. It will be important to determine the effects of flavonoids on CD45 tyrosine phosphatase. Protein tyrosine phosphorylation and calcium mobilization are strongly augmented by cross-linking CD4 or CD8 with CD3; this finding has implications for positive and negative thymic selection [21]. Since protein tyrosine phosphorylation is known to be affected by at least two flavonoids, genistein [20] and quercetin [22, 23], it seems likely that this fundamental process determining thymic selection may be a flavonoid-sensitive mechanism.



Flavonol Glycosides

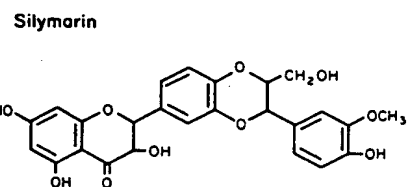
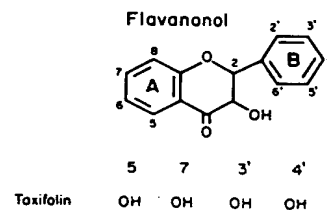
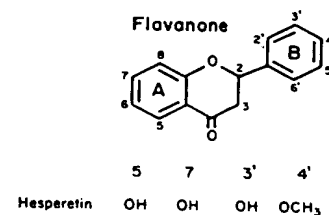
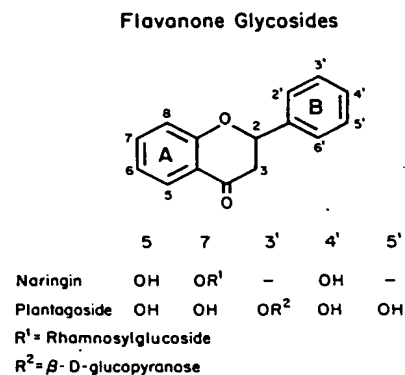
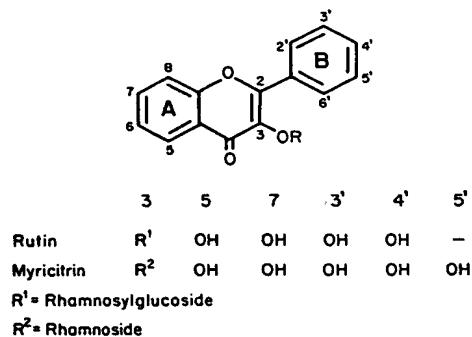


Fig. 1. Structures of various flavonoids.

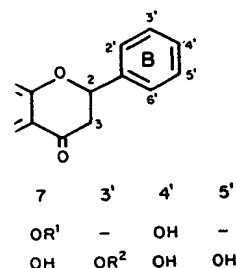
well as an effect on PLC effects on cell activation activity as a tyrosine portant to determine the 45 tyrosine phosphatase. orylation and calcium mgmented by cross-linking s finding has implications ymic selection [21]. Since ylation is known to be lavonoids, genistein [20] seems likely that this mining thymic selection e mechanism.

Phosphatidylinositol turnover is a central phenomenon in intracellular signal transduction, occurring in response to neurotransmitters, growth factors and hormones [24, 25]. Oncogene-induced transformation by *ras*, *src*, *erb*, *fms*, and *fes* also augments cellular PI turnover [cf. Ref. 26]. An important enzyme in PI turnover is PI kinase which phosphorylates the inositol moiety of PI on the 4-position and is referred to as phosphatidylinositol 4-kinase. It is of interest, therefore, that Nishioka and co-workers [26] found that the isoflavone, orobol, was a potent inhibitor of PI kinase from *Streptomyces* with an IC_{50} of 0.25 $\mu\text{g/mL}$; quercetin had an IC_{50} value of 1.8 and fisetin of 2.0 $\mu\text{g/mL}$. Kinetic analysis

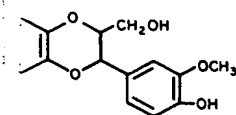
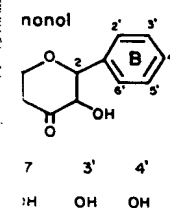
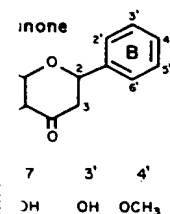
revealed that orobol is competitive with respect to ATP and uncompetitive with respect to PI. Psitectorigenin, another isoflavonoid related to genistein and orobol, proved to be a more active inhibitor of epidermal growth factor (EGF)-induced PI turnover in A431 cells with an IC_{50} of approximately 1 $\mu\text{g/mL}$ [27]. This compound inhibited PI turnover without affecting EGF receptor tyrosine kinase activity. Flavonoids with these biochemical properties should be useful probes in the functional analysis of PI turnover and its relationship to immune cell function.

In addition to PTK, protein kinase C (PKC), the ubiquitous Ca^{2+} and phospholipid-dependent,

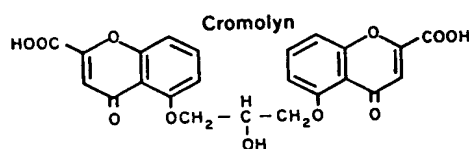
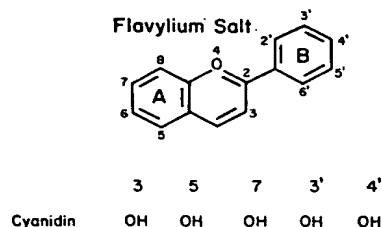
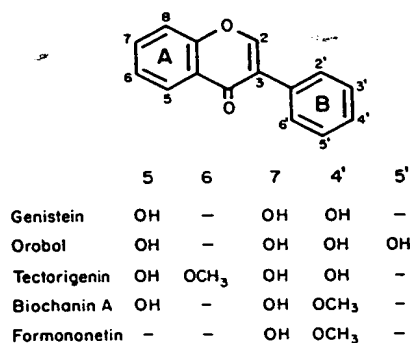
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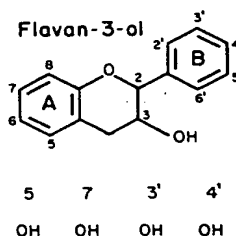
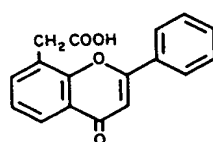
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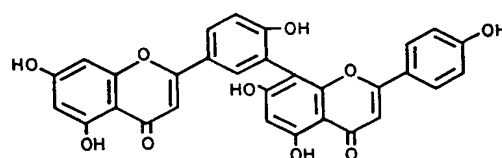
Isoflavone



Flavone Acetic Acid



Biflavonoid



Amentoflavone

Fig. 1 (continued).

multifunctional serine-threonine phosphorylating enzyme, which is involved in a wide range of cellular activities including tumor promotion and T lymphocyte function [15, 28], has also been shown to be inhibited *in vitro* by certain flavonoids [29-31]. Fisetin, quercetin and luteolin were the most active compounds in the study of Ferriola *et al.* [31]. An isoflavone congener of genistein, formononetin, was inactive. Fisetin was shown to competitively block the ATP binding site on the catalytic unit of protein kinase C [31].

Recently, Bagnasco *et al.* [32] studied transmembrane signaling by both CD3 and CD2 human T cell surface molecules and the involvement of protein kinase C translocation. T cell activation by monoclonal antibodies (mAbs) directed against both CD3/T cell receptor (CD3/TCR complex) and the CD2 molecule resulted in the rapid increase of intracellular ionized Ca^{2+} . Moreover, it was demonstrated in the human leukemic T cell line Jurkat that triggering with appropriate anti-CD2 mAbs induced the generation of IP_3 and DAG from the breakdown of PIP_2 . The appearance of such second messengers suggests that the CD2 molecule, like the CD3/TCR complex, may be linked to PLC. The investigators demonstrated that activation of Jurkat cells by anti-CD2 mAbs is also accompanied by translocation of PKC activity to the cell membrane in association with increased intracellular Ca^{2+} . By analogy with the effects of flavonoids on PTK, each of the steps in these experiments is potentially flavonoid-sensitive.

An important question is whether PTK activation is a prerequisite for PLC activation or whether these two pathways of signal transduction are regulated independently. It appears from experiments of June *et al.* [33, 34] that rapidly increased PTK activity is measurable prior to PLC activation (as determined by the appearance of IP_3) after T cell receptor complex ligation with anti-CD3 mAb. The PTK activity is sensitive to the effects of herbimycin, a benzoquinonoid ansamycin antibiotic which blocks oncogenic transformation by $\text{pp60}^{\text{v-src}}$. Mustelin and coworkers [35] obtained similar results, but they used the isoflavone genistein as an inhibitor of PTK. At concentrations which inhibited tyrosine phosphorylation of the TCR-zeta subunit, but not PLC activity (IP_3 increase), genistein blocked TCR-CD3-mediated activation of PLC, T cell proliferation and expression of IL-2 receptors. The effects were not related to genistein toxicity.

While all of these results clearly demonstrate that both tyrosine and serine-threonine kinases and PI kinase can be inhibited *in vitro* by certain flavonoids, more *in vivo* experiments remain to be done which would clearly show an effect on some facet of immune function. (A note of caution to all immunologists using experimental animals: the animals' chow contains flavonoids which could affect the outcome of various experimental manipulations. An experimentally ideal but unnatural diet would be an elemental one such as Vivonex.) The reported effects of flavonoids on the enzyme systems described above clearly suggest that they influence immune cell function. Several reports support this contention. In initial experiments, Mookerjee and co-workers

[36] demonstrated that both quercetin and tangeretin could depress the expression of class II histocompatibility (DR) antigens in human peripheral blood monocytes processing streptolysin O as antigen. The flavonoid effect was reversible. These investigators also observed that certain flavonoids reversibly inhibit lymphocyte proliferative responses to phyto mitogens, soluble antigens and phorbol esters by blocking an early event (or events) that follows exposure to the stimulus. Furthermore, quercetin and tangeretin were found to inhibit thymidine transport in stimulated lymphocytes. The finding that a flavonoid such as quercetin inhibits lymphocyte uptake of thymidine confirmed earlier reports of Graziani and Chayoth [37] and raises an important point: studies of inhibitors of DNA synthesis using [^3H]thymidine uptake must have controls determining the effect of the inhibitor on thymidine uptake itself.

Ishikawa *et al.* [38] studied the possible involvement of quercetin in tumor cell immunity. After exposure of the metastatic tumor BMT-11 I-9 cells (a clone of BMT-11, a transplantable mouse fibrosarcoma) to quercetin and cloning, clones were obtained which spontaneously regressed in normal syngeneic hosts. Possible mechanisms of regression of the regressor clones were studied by measuring cytotoxic T lymphocyte activity generated during mixed lymphocyte/tumor cell culture of spleen cells obtained from tumor-bearing mice. These studies show the potential ability of flavonoids to cause enzymatic alterations (prostaglandin formation) that may result in the production of tumor variants exhibiting modified immunological responses. Quercetin has inhibitory activity toward phospholipase A_2 whose product, arachidonic acid, is the essential substrate for the synthesis of prostaglandins.

Other studies suggest effects of flavonoids on immune responses. For example, tobacco glycoprotein (TGP), a polyphenol-rich, rutin-containing substance, can be purified from cured tobacco leaves and tobacco smoke condensate. One third of normal humans exhibit IgE-mediated reactivity to TGP. In mice, intradermal TGP stimulates a prolonged IgE response [39]. Similarly, rutin-derivatized bovine serum albumin (BSA) stimulates an IgE response to BSA but without hemagglutinating antibodies. The data suggested that rutin exerts a regulatory effect on isotype expression. Subsequently, it was shown that the polyphenol-containing substance stimulated IL-4 production by murine Th2 cells thus accounting for the augmented IgE formation [40].

In other experiments, Schwartz *et al.* [41, 42] described the effects of quercetin and several other flavonoids on the generation and effector function of cytotoxic lymphocytes. They showed that certain flavonoids inhibited the generation of cytotoxic lymphocytes in murine mixed spleen cell cultures and, as well, depressed their cytotoxic activity against P815 murine mastocytoma target cells. The addition of Cu^{2+} blocked the inhibition observed by certain flavonoids but not others, showing thereby that chelation of divalent cations such as Cu^{2+} cannot explain the action of all flavonoids in these systems. These experiments are of particular interest because

they demonstrate that two types of cognate cell-cell interaction can be affected by active flavonoids.

Several more reports indicate the capacity of selected flavonoids to affect immune responses. Yamada *et al.* [43] found that the flavanone glucoside, plantagoside, inhibited the *in vitro* immune response of mouse spleen cells to sheep RBC (SRBC) in a concentration-dependent manner. Plantagoside also inhibited the proliferative response of BALB/c spleen cells to the T cell mitogen concanavalin A but had no effect on the mitogenic activity of lipopolysaccharide or phytohemagglutinin (PHA). Also of interest is the fact that plantagoside is an α -mannosidase inhibitor; glycosidase inhibitors are useful probes in studies of inflammation, metastasis, transformation, and the immune response.

Delayed type hypersensitivity (DTH) reactions to dinitrofluorobenzene (DNFB), but not SRBC, were reduced in mice undergoing two-stage carcinogenesis: initiation with dimethylbenzanthracene (DMBA) followed by promotion with tetradecanoyl phorbol acetate (TPA). The reduced DTH response to DNFB appeared to be abrogated in mice treated with the flavonol glycosides mauritianin and myricitrin [44, 45]. A role for certain flavonoids in the maintenance of tumor immunity may be suggested by these experiments. Interestingly, the effects of flavonoid derivatives on TPA-induced inflammation [46] seem to be roughly parallel to their inhibitory activities on tumor promotion in mice [44].

A Hungarian team studied the hepatoprotective and immunomodulatory effects of systemically administered silymarin in patients with alcoholic cirrhosis. In addition to generally normalizing elevated liver enzymes, silymarin significantly increased the response of the peripheral blood lymphocytes of the subjects to stimulation with concanavalin A and PHA, while it decreased antibody-dependent cellular cytotoxicity and NK cell activity, and also reduced the percentage of T8+ cells in the peripheral blood [47]. More clinical investigations of this kind are badly needed in flavonoid research.

B lymphocytes

Cross-linking of B cell membrane immunoglobulin, the B cell antigen receptor, initiates the signal for B cell activation and maturation. B lymphocyte activation, like T cell activation, is accompanied by phosphorylation of tyrosine on particular B cell proteins [48-50]. In studies of human B cell precursors, Uckun *et al.* [51] found that IL-7 receptor ligation with rhIL-7 caused increased phosphorylation of tyrosine on multiple substrate proteins and stimulated phosphatidylinositol turnover with increased IP_3 generation (PLC activation) and also DNA synthesis. Genistein effectively abrogated the tyrosine kinase activity and the accompanying increase in IP_3 .

An example of ongoing, concurrent phosphorylation and dephosphorylation is seen in the experiments of Carter *et al.* [52] who studied tyrosine phosphorylation of PLC- γ 1 in L4 B-lymphoblastoid cells. From 0 to 30 min there was clear-cut evidence of phosphorylation followed by dephosphorylation

of several cellular proteins. These investigators also studied the PTK inhibitors genistein, tyrphostin and herbimycin. They found that genistein reduced the rise in cytosolic Ca^{2+} in B lymphocytes following ligation of membrane IgM and also observed the PTK dependence of PLC activation. It has also been demonstrated that protein tyrosine phosphorylation is induced in the Burkitt's lymphoma cell line Akata after stimulation with anti-IgG [53]. At noncytotoxic concentrations, genistein inhibited Epstein-Barr virus (EBV) activation as determined by the induction of EBV early antigen (and other antigens) and EBV early BZFI mRNA and its protein product ZEBRA. Biochanin A, another isoflavone, was also an active inhibitor of early antigen (EA) induction. Tumor promoter-stimulated induction of EA expression in EBV genome-carrying lymphoblastoid cells (Raji Cells) and the effects of flavonoids were studied by Okamoto *et al.* [54]. Quercetin (and retinol) caused an effective inhibition of EA expression while α -naphthylflavone, a synthetic flavonoid, had a weaker effect. Several other naturally occurring flavonoids were inactive. Since, in many systems, flavonoids are potent inhibitors of tumor promoter effects, one can speculate that certain flavonoids would also inhibit the enhanced expression of IL-2 receptors and immunoglobulin secretion stimulated by TPA as, for example, on sublines of an EBV-immortalized human B cell line as described by Polke *et al.* [55] and in keeping with the observations of Trevillyan *et al.* [17] with T cells.

Immunoglobulin secretion from mitogen-stimulated human peripheral blood B lymphocytes is, in fact, affected by quercetin. Cumella *et al.* [56] found that quercetin, but not taxifolin (dihydroquercetin), inhibited mitogen-stimulated immunoglobulin secretion of IgG, IgM and IgA isotypes *in vitro* with an IC_{50} of approximately 30 μ M for each isotype. Once again, in this secretory system, as in others (*vide infra*), quercetin was active while taxifolin was not.

Immunomodulatory effects of flavonoids: Interferon and NK cells

Recent reports indicate that flavone acetic acid, a synthetic flavonoid, exhibits dose-dependent *in vivo* antitumor activity against certain solid tumors in mice. The mechanism of action of flavone acetic acid is intriguing: the compound augments murine NK cell activity *in vivo* and this effect was found to be due to induction of interferon- α synthesis [57, 58]. Spleen cells of flavone acetic acid-treated mice demonstrated rapid expression of interferon- α mRNA [58]. Upregulation of interferon mRNA was also detected early following the administration of flavone acetic acid to mice. The flavone acetic acid effect was selective since no upregulation of splenic mRNA for interferon- β , IL-1 α or - β or IL-2 was detected after administration of flavone acetic acid. These data clearly indicate that flavone acetic acid stimulated gene activation. Another dimension of flavone acetic acid antitumor activity is its ability to cause vascular shutdown in the tumors. This effect can be attributed to the rapid induction of tumor necrosis factor (TNF); pretreatment with anti-TNF antibody abrogated the drug effect [59, 60]. It is tempting to speculate that naturally occurring dietary

flavonoids may also induce interferon synthesis and thereby act as anti-tumor agents.

NK cell cytotoxic activity against NK-sensitive tumor target cells K562 and U937 is accompanied by early increased incorporation of ^{32}P into PI, suggesting activation of phospholipase C [61]. A high quercetin concentration (100 μM) profoundly inhibited the increased PI metabolism and also inhibited killing activity. Other studies established that T lymphocyte and NK cell cytotoxic activity rely on extracellular Ca^{2+} . Ng *et al.* [62] studied the Ca^{2+} dependence using quercetin and Ca^{2+} channel antagonists. Quercetin inhibited Ca^{2+} -dependent killing. Cytolysis could be induced by simultaneous stimulation with TPA and ionophore A23187, suggesting that protein kinase C activation is involved. The inhibitory effect of quercetin could be through its action on PKC [31].

Macrophages and monocytes

Few studies on the effects of flavonoids on macrophage function have appeared. Oxyl radical generation by peripheral blood monocytes was suppressed by catechin as noted by Berg and Daniel [63]. A synthetic lipophilic derivative, 3-palmitoyl-(+)-catechin, enhanced the phagocytic activity of guinea pig Kupfer cells *in vivo* according to Piazza *et al.* [64]. As described earlier, Mookerjee and co-workers [36] demonstrated an effect of selected flavonoids on antigen presentation by human peripheral blood mononuclear cells. This observation should be confirmed as it has important implications for the earliest stages of the immune response.

The synthesis of IL-2 and leukotriene B (LTB_4) by human peripheral blood mononuclear cells was studied by Atluru *et al.* [65]. At a noncytotoxic concentration, genistein inhibited PHA-induced cell proliferation and IL-2 formation. The isoflavone also blocked LTB_4 generation in A23187-stimulated cells while H-7, a protein kinase C inhibitor, had no effect. LTB_4 formation in carragenin-induced intrapleural exudates in rats was reduced by intraperitoneal injection of quercetin and quercitrin but not by apigenin or luteolin, both of which lack a 3-position hydroxyl group (present in quercetin).

Studies of adhesion among human mononuclear leukocytes were reported by Patarroyo and Jondal [66]. The investigators studied the effect of phorbol ester-induced adhesion among human mononuclear leukocytes, the requirement for extracellular Mg^{2+} , and the effects of inhibitors of protein kinase C, lipoxygenase and ATPase. It was found that quercetin significantly inhibited phorbol 12,13-dibutyrate-induced cell aggregation/adhesion. The authors attributed the quercetin effect to inhibition of cellular ATPases but it is alternatively possible that the effect of quercetin could be due to its activity as an inhibitor of lipoxygenase and/or protein kinase C as described elsewhere in this report.

Mast cells and basophils

Mast cells play a central role in the pathogenesis of diseases such as allergic asthma, rhinoconjunctivitis, urticaria, anaphylaxis and systemic mastocytosis, and may well be important players in other chronic

inflammatory disorders such as Crohn's disease, and other varieties of inflammatory bowel disease, vasculitis, rheumatoid arthritis and others. Basophils are perhaps emerging as an important cell in the pathogenesis of late phase allergic reactions [67].

Both mast cells and basophils possess high affinity receptors for IgE in their plasma membranes. Cross-linking of these receptors is essential to trigger the secretion of histamine and other preformed, granule-associated mediators and to initiate the generation of newly formed phospholipid-derived mediators. Various flavonoids have been shown in a number of systems to influence this secretory process, most frequently as inhibitors. Definitive evidence of flavonoid regulation of secretion was first provided by Fewtrell and Gomperts [68, 69] and Bennett *et al.* [70] in studies of the secretion of histamine from rat mast cells stimulated with antigen, mitogen, or the divalent cation ionophore A23187 and the release of β -glucuronidase from stimulated rabbit leukocytes. Quercetin, kaempferol and myricetin were found to inhibit the release of rat mast cell histamine. Following these reports, Middleton and co-workers [71, 72] undertook an examination of the effect of a number of naturally occurring flavonoids on the secretion of histamine from human basophils. Quercetin inhibition of antigen-stimulated human basophil histamine release [71] was found to be concentration-dependent, instantaneous in onset of action, antagonistic to the histamine release-augmenting effect of D_2O , not affected significantly by increased extracellular Ca^{2+} concentrations, and not enhanced by theophylline (suggesting that inhibition is not a cyclic AMP-dependent process). Subsequent experiments revealed critical structure-activity relationships governing the flavonoid effect on antigen-induced histamine release [72]. Inhibition of antigen-induced histamine release was associated with the following structural features: the presence of a C4 keto group, a reduced double bond at position C2-C3 in the γ -pyrone ring, and an appropriate pattern of hydroxylation in the B ring. Flavonoid glycosides, rutin and naringin, were inactive as were the flavanones (reduced C2-C3 bond), taxifolin and hesperitin. Morin, catechin and cyanidin were also inactive. Polymethoxylated compounds such as nobiletin and tangeretin showed less or no inhibitory activity against antigen-induced histamine release (as compared to their activity as inhibitors of lymphocyte activation) [36].

Further studies were undertaken to determine the effect of flavonoids on basophil histamine release stimulated by anti-IgE and concanavalin A (IgE-dependent histamine releasing agents); the chemo-attractant peptide, f-MetLeuPhe, and the tumor promoter phorbol ester, TPA (both f-MetLeuPhe and TPA are receptor-dependent histamine-releasing agents); and the divalent cation ionophore A23187 (bypasses receptor-dependent processes and carries Ca^{2+} directly into the cytoplasm). The results showed that the histamine-releasing effect of each of these secretagogues could be inhibited by some, but not all, of eleven flavonoids representing five different chemical classes [73]. The nature of the stimulus for histamine release and the structure of specific flavonoids appeared to determine whether a

particular compound would exert inhibitory activity. The results suggest that each of the secretagogues may utilize a different pathway of cell activation and that these pathways may be differentially sensitive to the action of particular flavonoids. It is not surprising, perhaps, to record that yet another stimulus of basophil histamine release, i.e. histamine releasing factor, can be inhibited by quercetin [74]. The effect of quercetin to uniformly inhibit basophil histamine secretion stimulated by a variety of agonists strongly suggests that there is a final common pathway utilized by each of these agonists which is sensitive to quercetin and other structurally appropriate flavonoids. A number of other investigators have also described inhibition of histamine release from mast cells by certain flavonoids [75-77], including some structurally unique flavonoid dimers such as amentoflavone (a biapigenin).

It is important to emphasize that the effects of active flavonoids on basophil histamine secretion are reversible, to wit, a basophil-containing cell suspension can be exposed to quercetin (50 μ M) for 30 min and then washed twice, resuspended, and found to respond normally to antigen. However, if the histamine secretory reaction is initiated and an active flavonoid such as quercetin is added at 2, 5, 10 or 15 min after addition of antigen, there is at each time point an immediate cessation of further release of histamine [71]. These observations indicate that antigen activation of basophils results in the generation of a flavonoid-sensitive substance(s), interaction of which with the flavonoid strikingly alters the outcome of the activation process, in this case histamine secretion from basophils. The nature of the flavonoid-reactive substance(s) is unknown.

Other evidence suggests that calmodulin may be involved in the mechanism of secretion of histamine from granules of mast cells and basophils [78]. It is of interest, therefore, that quercetin appears to interact with the Ca^{2+} -calmodulin complex with resultant inactivation of Ca^{2+} -calmodulin-dependent activities, including the effects of tumor promoters [79-81].

A number of flavonoids possess lipoxygenase inhibitory activity [4, 82]. Marone *et al.* [83] showed that basophil histamine release was inhibited by eicosatetraynoic acid, a chemically unique lipoxygenase inhibitor, and suggested that a lipoxygenase-derived product of arachidonic acid metabolism may be required for basophil histamine release. Interestingly, many flavonoid inhibitors of histamine release are also good lipoxygenase inhibitors. Several flavonoids are relatively selective inhibitors of 5-lipoxygenase which initiates the biosynthesis of leukotrienes, compounds considered to be of importance in mediator release, inflammation and immediate hypersensitivity pathophysiology in tissues undergoing allergic reactions [84]. Cirsiliol (3',4',5-trihydroxy-6,7-dimethoxy flavone) is a potent inhibitor and caused 97% inhibition of the enzyme partially purified from rat basophilic leukemia cells. At 10 μ M the compound caused 99% suppression of immunologic release of leukotrienes (SRS-A) from passively sensitized guinea pig lung (IC_{50} approximately 0.4 μ M) [82].

Stimulation of Ca^{2+} -dependent protein phos-

phorylation during secretagogue-induced exocytosis in rat mast cells was described by Sieghart and co-workers in 1978 [85]. Purified rat peritoneal mast cells which had been labeled with ^{32}P and then stimulated by addition of compound 48/80 or ionophore A23187 resulted in the phosphorylation of four proteins of apparent molecular weights 78,000, 68,000, 59,000 and 42,000. Phosphorylation of the proteins with apparent molecular weights of 68,000, 59,000 and 42,000 was evident within 10 sec after addition of 48/80 whereas phosphorylation of the 78,000 molecular weight protein was not evident until 30-60 sec after addition of the secretagogue. The experiments clearly indicated that the secretory response of the mast cell is associated with protein phosphorylation and that regulation of exocytosis is related to protein phosphorylation. In subsequent experiments [86] the authors briefly noted that quercetin and kaempferol (10 μ M), known inhibitors of rat mast cell histamine secretion, also increased the incorporation of radioactive phosphate into a single protein band with an apparent molecular weight of 78,000. The same group of investigators reported that the antiasthmatic drug cromolyn (chemically related to flavonoids) promotes the incorporation of radioactive phosphate into a single rat mast cell protein of apparent molecular weight 78,000 [87]. The finding provided an insight into the mechanism of inhibition by cromolyn of mast cell secretion triggered by an immunologic stimulus, anti-rat IgE. Whether the PTK family of kinase enzymes is involved in mast cell or basophil secretory responses has not been established, but Sagieisenberg *et al.* [88] have demonstrated the involvement of PKC in rat basophilic leukemia cell histamine secretion.

A still unresolved question is just what cellular component in activated mast cells or basophils interacts with cromolyn (mast cells) or active flavonoids (mast cells and basophils) to inhibit the secretory process. Fewtrell and Gomperts [69] and Middleton *et al.* [71] demonstrated that only activated mast cells or activated basophils are affected by quercetin and other inhibitory flavonoids (i.e. the unstimulated cells can be exposed to the flavonoids, be washed, and subsequently shown to react normally to a secretagogue with histamine release). Fewtrell and Gomperts [69] also observed that pretreatment of rat mast cells with cromolyn (30 μ M) for 30 min completely abolished the inhibition normally observed upon subsequent exposure to quercetin (30 μ M) added together with antigen. This suggests that cromolyn and quercetin act at the same molecular site.

Another dimension of the exocytotic process and cromolyn/flavonoid activity is the possible relevance of the Ca^{2+} -ATPase membrane transport regulator. Certain flavonoids, notably quercetin, interfere with the activity of membrane transport ATPases including the Ca^{2+} -dependent ATPase which is one of the intrinsic cellular mechanisms that maintains low cytosolic Ca^{2+} concentrations. Fewtrell and Gomperts [68] found a very good correlation between the ability of certain flavonoids to inhibit rat mast cell histamine secretion and inhibition of Ca^{2+} -dependent ATPase activity. They suggested that the

effect of quercetin to inhibit secretion from stimulated cells was due to its inhibitory effect on plasma membrane Ca^{2+} -ATPase. Racker [89] has suggested that the transport ATPases of cell membranes with their associated ion flux pathways (channels) are separate structural entities which when properly coupled constitute the ATP-dependent ion pumps. Some flavonoids including quercetin inhibit aerobic glycolysis and growth of certain tumor cells by specifically modifying or "repairing" a defective control in the ATPase transport system [90].

Neutrophils

The inhibitory effect of flavonoids on secretory processes is not limited to basophils and mast cells. Bennett *et al.* [70] showed that a number of flavonoids were capable of inhibiting stimulated rabbit neutrophil lysosomal enzyme release. Also, Berton and co-workers [91] and Schneider *et al.* [92] reported that concanavalin A-induced secretion of lysosomal enzyme from polymorphonuclear leukocytes of albino guinea pigs and healthy human volunteers was inhibited by quercetin (which did not have any effect on the binding of concanavalin A to the cell membrane receptors). Rutin and morin were inactive, in keeping with the findings of the human basophil experiments.

Phagocytosis is accompanied by a dramatic increase in oxygen consumption (respiratory burst) with an attendant production of reactive oxygen intermediates such as the relatively less active superoxide anion and the various oxidizing species (hydrogen peroxide, hydroxyl radical and the hypohalites) by neutrophils [93]. These are generated sequentially starting with superoxide anion production by a membrane-bound NADPH oxidase activity [93]. The highly reactive oxygen metabolites along with other mediators elaborated by neutrophils and macrophages promote inflammation and cause tissue damage [94]. Oxidant release (as assayed by the production of luminol-dependent chemiluminescence) by human neutrophils was shown to be inhibited by certain flavonoids [95], an effect possibly related to inhibition of the 5-lipoxygenase pathway of arachidonic acid metabolism [96]. T Hart *et al.* [97] recently reported a similar inhibitory effect of different flavonoids on the production of reactive oxygen species by activated human neutrophils using the above chemiluminescence method. Four selected flavonoids inhibited myeloperoxidase (MPO) release, while two of these strongly inhibited this activity. Considering luminol-dependent chemiluminescence production by neutrophils to be an MPO-dependent process, these authors contend that these effects may mask the effects of flavonoids on oxidant production. Using the luminescent probe lucigenin for the exclusive detection of superoxide anion release, T Hart *et al.* [97] showed that the release of this species by human neutrophils was inhibited by flavonoids. Essential determinants for inhibition of superoxide anion release appear to be the OH groups located in the B-ring of the flavonoid molecule. The formation of superoxide anion is dependent on the activation of NADPH oxidase localized in the plasma membrane, which is subject to flavonoid inhibition [98].

In addition to inhibiting the activity of a purified human neutrophil MPO, quercetin was also found to depress this activity in a system employing intact human neutrophils [99]. In this case, in inhibiting the activity of the purified MPO, quercetin was significantly more potent than methiomazole, a known specific inhibitor of MPO [100]. In addition, quercetin was found to have an ability to directly scavenge hypochlorous acid (HOCl), a highly reactive chlorinated species generated by the MPO- H_2O_2 -Cl system [99]. The inhibition of neutrophil MPO activity by flavonoids can result in the impairment of oxidant production. Such impairment could diminish the formation of highly toxic hypochlorous acid and hypochlorite ion (OCl^-). A consequence of this would be a decrease in the inactivation of α -antitrypsin [101], which could result in the progressive inactivation of neutrophil-derived and other tissue-damaging proteolytic enzymes.

Quercetin was found to be a potent inhibitor of human neutrophil degranulation and superoxide anion production induced by different secretagogues [102, 103]. Quercetin also inhibited the phosphorylation of neutrophil proteins accompanying neutrophil activation by PMA. Phosphorylation of a specific neutrophil protein (mol. wt 67,000) was reported to be particularly sensitive to quercetin at concentrations that also diminished neutrophil degranulation and superoxide production, suggesting thereby that the phosphorylation of this particular protein is an important intracellular event associated with neutrophil activation [102].

In other studies, Lee *et al.* [104] examined the effect of quercetin on the release of β -glucuronidase from human neutrophils stimulated with opsonized zymosan and found that quercetin inhibited the release of β -glucuronidase although the effect was not strong. However, it was also found that the release of [^3H]arachidonic acid from prelabeled neutrophils was also inhibited by quercetin, strongly suggesting an inhibitory effect of the flavonoid on phospholipase A_2 in keeping with the findings of Lanni and Becker [105].

Experiments performed by Busse and co-workers [95] showed that quercetin and chalcone were slightly effective inhibitors of neutrophil β -glucuronidase secretion stimulated by opsonized zymosan. These investigators also described that quercetin and the other flavonoids were quite effective inhibitors of opsonized zymosan-stimulated generation of superoxide anion and the chemiluminescence phenomenon. Long and co-workers [106] found that quercetin had at least three separate effects on human polymorphonuclear leukocytes: (1) quercetin inhibited the Mg^{2+} -dependent ecto-ATPase in a noncompetitive fashion, (2) it inhibited O_2 consumption, glucose oxidation and protein iodination in cells exposed to opsonized zymosan and TPA, and (3) it inhibited transport of the nonmetabolizable glucose analog, [^3H]2-deoxyglucose.

Eosinophils

Ionophore A23187-induced eosinophil degranulation with release of Charcot-Leyden crystal protein and eosinophil cationic protein is inhibited by

activity of a purified β -glucuronidase was also found employing intact cells. In inhibiting β -glucuronidase, quercetin was as effective as methimazole, a known inhibitor [100]. In addition, quercetin inhibited directly (OC1), a highly active enzyme. Such impairment of β -glucuronidase of highly toxic cells (OC1⁻). A decrease in the activity of this enzyme could result in neutrophil-derived toxic enzymes. Quercetin is a potent inhibitor of β -glucuronidase and superoxide anion secretagogues inhibited the phosphorylation of β -glucuronidase (wt 67,000) was inhibited by quercetin at a concentration of 10 μ M. This finding suggests that quercetin inhibits β -glucuronidase, suggesting that this particular event associated

examined the β -glucuronidase with opsonized cells inhibited the effect was found that the β -glucuronidase strongly inhibited the findings of

and co-workers were slightly β -glucuronidase zymosan. These quercetin and the active inhibitors generation of luminescence [106] found that quercetin effects on: (1) quercetin to-ATPase in inhibited O_2 protein iodide zymosan and export of the $[^3H]2$ -deoxy-

ophol degranulation crystal protein inhibited by

quercetin, but not by taxifolin (dihydroquercetin), in a concentration-dependent manner [107]. Thus, the activated eosinophil responds to these flavonoids in the same fashion as other secretory cells, e.g. basophils and mast cells. Whether eosinophil degranulation stimulated by other immunologic or nonimmunologic stimuli such as allergen or platelet-activating factor (PAF), for example, will be inhibited by selected flavonoids remains to be determined but it is of interest that PI hydrolysis accompanies stimulation of murine eosinophils with anti-IgG F(ab')₂ acting through an Fc- γ RII mechanism. This process is neomycin sensitive (inhibition of phospholipase C) and is required for 5-lipoxygenase activation and LTC₄ generation [108].

Platelets

In addition to their role in hemostasis and thrombosis, considerable evidence implicates platelets as inflammatory cellular elements [109]. Platelets are also key participants in atherogenesis. A number of proinflammatory mediators are derived from platelets including thromboxane A₂, PAF, and serotonin as well as transforming growth factor- β , platelet-derived growth factor and lipoxygenase metabolites. In light of the above, it is of interest that a number of flavonoids significantly affect platelet function. This subject has been reviewed in detail [110]. Flavonoids inhibit platelet adhesion, aggregation and secretion. Of the variety of compounds studied, the most active inhibitors of platelet function *in vitro* were effective in the 1–10 μ M range. Certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase and this may be in part the explanation for their ability to inhibit platelet function. Flavonoids have been related to the inhibition of arachidonic acid metabolism by cyclooxygenase [111]. The inhibition by flavonoids of platelet activation by thromboxane-dependent stimuli may be related to this effect. The effect of selected flavonoids on platelet aggregation/adhesion is akin to their effect on mononuclear cell adhesion as described earlier and is another example of their potential capacity to regulate the activity of adhesion molecules [112].

Genistein apparently can affect cell function in ways possibly unrelated to protein tyrosine phosphorylation [113]. For example, platelet tyrosine phosphorylation stimulated by thrombin was only weakly affected by genistein but it inhibited platelet aggregation and serotonin secretion. On the other hand, this isoflavone suppressed platelet aggregation, serotonin secretion and protein tyrosine phosphorylation triggered by collagen and stable thromboxane A₂ analogs. These investigators also provided some insight into isoflavone structure-activity relationships: daidzein, an isoflavone lacking a 5-position hydroxyl group, is inactive as a PTK inhibitor but is capable, like genistein, of inhibiting binding of the stable thromboxane A₂ analog, U46619, to platelets with associated reduction in collagen- or U46619-induced platelet responses [113].

Gryglewski and coworkers [114] studied the mechanism of the antithrombotic action of flavonoids. Quercetin and rutin were capable of dispersing

platelet thrombi adhering to rabbit aortic endothelium *in vitro* and prevented platelets from aggregating over a blood-superfused collagen strip (adhesion-related phenomena). The *in vivo* counterpart of these experiments involved the infusion of quercetin and rutin into an extracorporeal stream of blood. Quercetin and rutin inhibited the deposition of platelet thrombi on the blood-superfused collagen strip at calculated plasma concentrations of 0.05 and 0.03 μ M. Analogously, in the model for studying platelet-endothelium interactions, quercetin and rutin, when infused into the stream of blood which superfused a rabbit aortic endothelial surface, caused the disaggregation of preformed platelet thrombi, again at low concentrations. Clearly the expression and/or activity of adhesion molecules are affected by the flavonoids. The authors concluded that flavonoids are antithrombotic because they are bound selectively to mural platelet thrombi and, because of their free radical scavenging properties, modify damaged endothelial cells and permit normal prostacyclin and endothelium-derived relaxing factor synthesis [114]. The relevance of these experiments to immunologic and allergic tissue injury is once again related to the possibility that platelets may be activated and involved in some of these disorders.

PAF is a well recognized proinflammatory mediator derived from membrane phospholipids by the enzymatic activity of phospholipase A₂ and an acetyl transferase. PAF receptor-coupled activation of phosphoinositide-specific phospholipase C and phosphorylation of several cellular proteins has been reported. Dhar and colleagues [115] used the isoflavonoid, genistein, to investigate the possible involvement of tyrosine kinase in PAF-stimulated platelets and the relationship between protein phosphorylation and PLC activation. PAF alone stimulated PLC activity as measured by the production of IP₃ and genistein at the high concentration of 0.5 mM decreased PAF-stimulated PLC activity to control levels. At this concentration genistein also blocked PAF-stimulated platelet aggregation. In addition, genistein also reduced PAF-induced phosphorylation of proteins of molecular weights 20,000 and 50,000. Moreover, polymorphonuclear leukocyte-induced 40,000 molecular weight protein phosphorylation was additionally affected by genistein. Taken together these results strongly suggest that genistein inhibited PTK at an early stage of signal transduction resulting in inhibition of (or associated with inhibition of) PLC which in turn would result in decreased activation of protein kinase C via reduced PLC-catalyzed formation of DAG. The combined effects would, therefore, result in a reduction of protein phosphorylation. Based on these and other experiments the authors finally concluded that tyrosine phosphorylation is involved in the PAF receptor-coupled activation of PLC and signal transduction mechanism. It is tempting to speculate that there may be other isoflavonoid or flavonoid compounds, both natural and synthetic, which could affect the outcome of PAF-stimulated pathological states.

Conclusion

In summary, no doubt can remain that the

flavonoids have profound effects on the function of immune and inflammatory cells as determined by a large number and variety of *in vitro* and some *in vivo* observations. That these ubiquitous dietary chemicals may have significant *in vivo* effects on homeostasis within the immune system and on the behavior of secondary cell systems comprising the inflammatory response seems highly likely but more work is required to strengthen this hypothesis.

Ample evidence indicates that selected flavonoids, depending on structure, can affect (usually inhibit) secretory processes, mitogenesis, and cell-cell interactions including possible effects on adhesion molecule expression and function. The possible action of flavonoids on the function of cytoskeletal elements is suggested by their effects on secretory processes. Moreover, evidence indicates that certain flavonoids may affect gene expression and the elaboration and effects of cytokines and cytokine receptors.

How all of these effects are mediated is not yet clear but one important mechanism may be the capacity of flavonoids to stimulate or inhibit protein phosphorylation and thereby regulate cell function. Perhaps the counterbalancing effect of cellular protein tyrosine phosphatases will also be found to be affected by flavonoids. Some flavonoid effects can certainly be attributed to their recognized antioxidant and radical scavenging properties. A potential mechanism of action that requires scrutiny, particularly in relation to enzyme inhibition, is the redox activity of appropriately configured flavonoids.

Finally, in a number of cell systems it seems that resting cells are not affected significantly by flavonoids but once a cell becomes activated by a physiological stimulus a flavonoid-sensitive substance is generated and interaction of flavonoids with that substance dramatically alters the outcome of the activation process.

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Soybean Isoflavones. Characterization, Determination, and Antifungal Activity

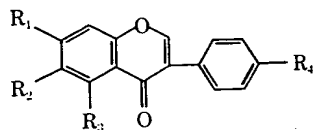
Michael Naim, Benjamin Gestetner, Shmuel Zilkah, Yehudith Birk,* and Aron Bondi

The isolation and separation of defatted soybean meal isoflavone glycosides by chelation with aluminum oxide and subsequent chromatography on polyamide columns have been described. A procedure for the quantitative determination *via* gas chromatography of isoflavones in soybean meal was elaborated. The resulting analytical data showed that the content of isoflavones in soybeans amounts to 0.25% and that 99% of the isoflavones are present as glycosides: 64% genistin,

23% daidzin, and 13% glycitein 7-*O*- β -glucoside. Soybean oil does not contain isoflavones. The growth depression of *Trichoderma lignorum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium spp.*, *Rhizopus spp.*, and *Sclerotium rolfsii* exerted by soybean isoflavones is due mainly to the presence of free isoflavones in the growth medium, while the activity of isoflavone glycosides is in most instances negligible.

Soybeans have been known to contain the two isoflavone glycosides genistin and daidzin and their respective aglycones (Walz, 1931; Walter, 1941). Evidence has been provided recently on the presence of a third isoflavone, glycitein, the structure of which has been determined (Naim *et al.*, 1973).

The structures of the known soybean isoflavones are as follows.



genistein, $R_1 = \text{OH}$; $R_2 = \text{H}$; $R_3 = \text{OH}$; $R_4 = \text{OH}$
 genistin, $R_1 = \text{O-glucosyl}$; $R_2 = \text{H}$; $R_3 = \text{OH}$; $R_4 = \text{OH}$
 daidzein, $R_1 = \text{OH}$; $R_2 = \text{H}$; $R_3 = \text{H}$; $R_4 = \text{OH}$
 daidzin, $R_1 = \text{O-glucosyl}$; $R_2 = \text{H}$; $R_3 = \text{H}$; $R_4 = \text{OH}$
 glycitein, $R_1 = \text{OH}$; $R_2 = \text{OCH}_3$; $R_3 = \text{H}$; $R_4 = \text{OH}$
 glycitein 7- β -*O*-glucoside, $R_1 = \text{O-glucosyl}$; $R_2 = \text{OCH}_3$;
 $R_3 = \text{H}$; $R_4 = \text{OH}$

The wide use of soybeans as a protein source for humans and farm animals implies the quantitative assessment and evaluation of minor constituents, such as isoflavones at the levels normally ingested. It has been the aim of the present study to isolate and separate the native free isoflavones and isoflavone glycosides in soybeans and to elaborate a reliable analytical method for their quantitative determination. In view of the presence of other glycosides of genistein in different plants (Zemplen *et al.*, 1943; Szabo *et al.*, 1958), it was of interest to establish the position of glycosidic linkages in soybean isoflavone glycosides.

Since antifungal factors in red clover have been identified as isoflavones (Virtanen and Hietala, 1958; Bredenberg, 1961), and isoflavonoids were recognized as phytoalexins in various plants (Kuc, 1972), and also in infected soya plants (Keen *et al.*, 1971), it has been attempted to study the effect of soybean isoflavones on pathogenic fungi and to relate fungistatic activity to their composition and structure.

EXPERIMENTAL SECTION

Isolation, Separation, and Identification of Isoflavone Glycosides. Isoflavone glycosides were extracted on a preparative scale from an aqueous syrup (Chayot Industries, Ashdod, Israel). The syrup, which contains about 50% dry matter, was obtained as a residue after extraction

with 60% ethanol of commercially defatted soybean flakes and subsequent evaporation of the ethanol. Ten kilograms of soybean flakes yielded approximately 3 kg of syrup.

A portion of the syrup (3.5 l.) was stirred with two volumes of acetone for 2 hr at room temperature for extraction of the isoflavones. The isolation and separation of the isoflavone glycosides were carried out according to Naim *et al.* (1973). The glycosides were separated by chelation of those bearing an unsubstituted hydroxyl group at C_5 (such as genistin) with aluminum oxide G (Merck) (Wang, 1971). The nonchelated glycosides of daidzein and glycitein were washed out with 50% MeOH, filtered through Whatman paper 42, and separated by column chromatography as described later. The chelated glycoside (genistin) can be liberated from the cake by washing with 4% (w/v) HCl in methanol. Since the procedure did not enable full separation of genistin from accompanying aluminum oxide, pure genistin was obtained by recrystallization of the glycosides from 80% ethanol according to Walter (1941).

After evaporation of the solvent the nonchelated glycosides were separated by column chromatography on polyamide. Polyamide (Woelm) was mixed with an equal weight of Celite 545 and then equilibrated in ethyl acetate and poured into a 2.5 \times 70 cm column. A 200-mg portion of the nonchelated glycosides was suspended in a small amount of methanol and applied to the column. Elution was started with ethyl acetate and followed by solutions of 8 and 10% methanol in ethyl acetate. Fractions of 10 ml were collected and their absorbancy was measured at 260 nm. The fractions were evaporated to dryness and analyzed for isoflavones as described later.

Separation and Identification of Free Isoflavones. Individual glycosides, obtained as described above, or mixtures of glycosides were subjected to acid hydrolysis according to Naim *et al.* (1973). For analyses of isoflavone aglycones, tlc and glc were used.

Tlc was performed on Kieselgel G (Merck) with the following solvent mixtures: (A) chloroform-methanol (9:1) (Beck, 1964); (B) benzene-ethyl acetate-petroleum ether (bp 40-60 $^\circ$)-methanol (6:4:3:1) (Barz, 1969); (C) ether-petroleum ether (bp 40-60 $^\circ$) (7:3) (Barz, 1969).

The isoflavones on the chromatoplates were detected either with the Folin-Ciocalteu reagent in an ammonia-saturated chamber (Beck, 1964) or with the aid of an ultraviolet lamp at 366 nm (Beck, 1964). R_f values were quoted relative to genistein. Solvent C was used for preparative purposes by double development in the same direction.

Glc was performed on trimethylsilyl derivatives using a 2 m \times 0.52 cm column of 0.75% SE-30 on Gas Chrom Q at an operating temperature of 220 $^\circ$, as described by Lindner (1967). T_r values were quoted relative to formononetin.

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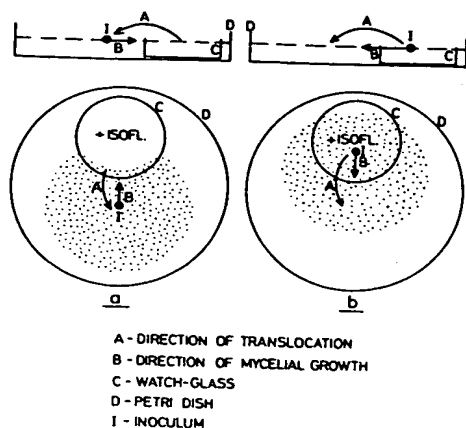


Figure 1. Schematic presentation of translocation of isoflavone aglycones in *R. solani*.

Characterization of Isoflavones. Uv spectra were determined in a Beckman DB-G spectrophotometer. Spectroscopic grade methanol was used as solvent. The effects of NaOMe, $\text{AlCl}_3\text{-HCl}$, NaOAc, and NaOAc- H_3BO_3 on the uv spectra were examined as described by Mabry *et al.* (1970).

Analysis of the Carbohydrate Moiety. The configuration and constitution of the carbohydrate moiety and its linking position to the aglycone were determined by methylation of separated isoflavone glycosides according to the procedure of Kuhn *et al.* (1955). After two successive methylations, methanolysis was carried out in 4% (w/v) methanol-HCl for 6 hr. Glc of the methylated sugars was performed on 2 m \times 0.32 cm columns of Chromosorb W (AW), coated with 5% (w/w) neopentyl glycol adipate polyester at an operating temperature of 150° (Aspinall *et al.*, 1968). T_r values are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl- β -D-glucopyranoside.

Quantitative Determination of Isoflavones in Soybeans. For this determination 250 g of the finely ground soybean flour (Wayne variety, 1969 crop) was exhaustively extracted first with ether and then with absolute methanol in a Soxhlet apparatus. After evaporation of the ether, the free isoflavones present in the oil were extracted with three portions (100 ml each) of absolute methanol. The methanolic extracts of the oil as well as of the soybean flour were concentrated to a syrup. A 50-g portion of the soybean flour residue (after extraction with ether and methanol) was subjected to acid hydrolysis in 1 N H_2SO_4 in dioxane-water (1:3), for 10 hr and the liberated isoflavones were extracted with ether. The three extracts, i.e., the methanol extracts of the oil, the methanol extract of the soybean flour, and the ether extract of the acid hydrolysate of the residual flour, were subjected to polyamide column chromatography. Elution was performed with ethyl acetate and then by solutions of 5, 7 (for aglycones), and 10% methanol (for glycosides) in ethyl acetate successively. Fractionation was followed up by measuring the absorbancy of the fractions at 260 nm, and the fractions were then evaporated to dryness. The fractions which contained isoflavones were analyzed by glc according to Lindner (1967). The free isoflavone content of the fractions which were high in lipids could not be determined by glc, since they contained impurities, which had retention times identical with those of isoflavones. The amount of free isoflavones was determined therefore by the tlc method of Beck (1964).

Effect of Isoflavones on Growth of Fungi. Isoflavone effect was determined by either of two methods. (a) *Determination of Dry Weight as a Measure of Growth.* *R. solani* was grown in 20 ml of growth medium which con-

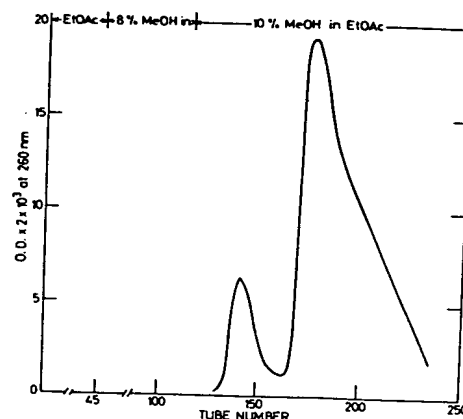


Figure 2. Separation of glycitein 7-*O*- β -glycoside and daidzin by chromatography on a polyamide column.

tained 0.5% yeast extract (Difco), 0.5% Bacto-peptone (Difco), and 2% glucose. After 48 hr, disks (5 mm diameter) of mycelium were cut from the margins of the colony and transferred to flasks containing 20 ml of yeast extract broth supplemented with 0.1% of soybean isoflavones. The flasks were incubated at 28° in a shaking water bath adjusted to 100 strokes/min. Mycelial growth was allowed to proceed for 72 hr, and flasks were withdrawn at 24-hr intervals (five replicates each time). The mycelia of each flask were separated from the growth medium by washing on a Buchner funnel, then transferred to previously dried and weighed petri dishes and dried at 90° until they reached constant weight.

(b) *Measurement of Mycelial Growth of Various Fungi.* The effect of isoflavones on mycelial growth was assayed on the following fungi: *Trichoderma lignorum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium spp.*, *Rhizopus spp.*, and *Sclerotium rolfsii* (received from the Department of Microbiology and Phytopathology, the Faculty of Agriculture, Rehovot, Israel). The fungi were grown on potato dextrose agar (PDA), according to Johnson *et al.* (1959), in petri plates (85 mm in diameter) with 15 ml of medium. Disks (5 mm in diameter) of agar with mycelium were cut from the margins of 48-hr-old colonies with the aid of a sterile cork-borer and transferred to petri plates containing PDA supplemented with various amounts of isoflavones. Mycelial growth was allowed to proceed at 28° until the mycelium in the control reached the margin of the petri plate. Each experiment was carried out in five replicates, and mycelial growth of each fungus was determined by the area covered by the mycelium.

Liberation of aglycones from their glycosides into the growth medium was followed by tlc (Beck, 1964). The effect of methylated free isoflavones on *R. solani* was also examined. A mixture of methylated free isoflavones was prepared by treating the mixture of free isoflavones with CH_2N_2 in ether during 3 days. Absorption and translocation of free isoflavones by *R. solani* were studied by the "dish technique" as described by Schutte (1956) (Figure 1).

RESULTS AND DISCUSSION

Characterization of Soybean Isoflavone Glycosides. The results obtained by separation of the nonchelated glycosides on a polyamide column are shown in Figure 2. After acid hydrolysis of the fractions glc and tlc analyses of the aglycones revealed that 10% methanol in ethyl acetate elutes glycitein 7-*O*- β -glycoside which is followed by daidzin. When genistin is not removed by chelation prior to column chromatography, it is also eluted with the same solvent and overlaps the peak of glycitein 7- β -*O*-glucoside. A very good separation between the previously nonsepara-

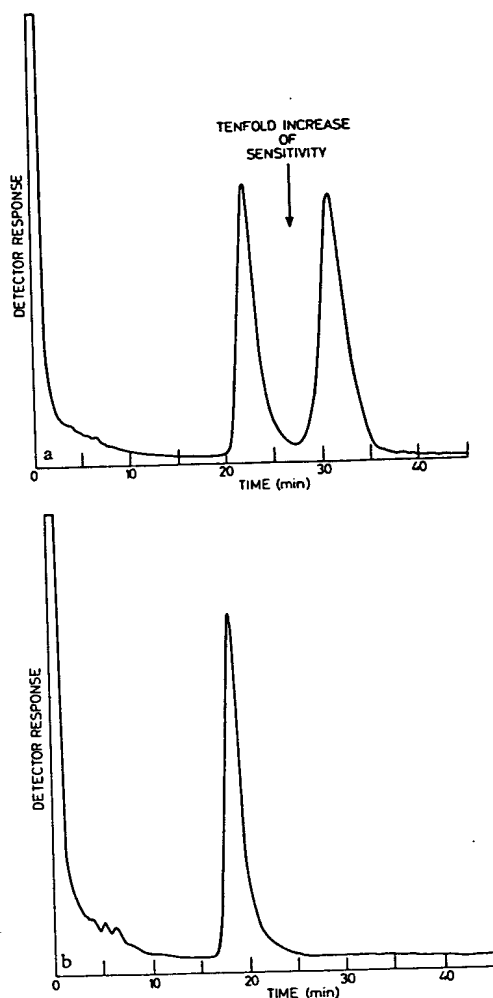


Figure 3. Gas-liquid chromatographic separation of free isoflavones, obtained by acid hydrolysis of their glycosides, eluted from a polyamide column with 10% MeOH in EtOAc: (a) first fraction; (b) second fraction.

ble genistin and daidzin could be achieved by this means (Figures 3a and b). Recovery tests, conducted with a mixture of known amounts of the glycosides, gave yields of 85–86% from the column.

The aglycones in the hydrolysates of mixtures of glycosides were identified by tlc and glc (Table I). The per cent composition of the mixtures was 87.5% genistein, 10% daidzein, and 2.5% glycitein. No additional isoflavones were present.

Gas chromatographic analysis of the separated methylated glycosides showed the presence of methyl 2,3,4,6-tetra-*O*-methylglucopyranoside as the only sugar derivative. This finding excludes the possibility of a carbohydrate chain attached to the aglycone, but it does not rule out the attachment of several glucose residues, each at another point, to the aglycone (Zemlen *et al.*, 1943; Szabo *et al.*, 1958). This possibility was, however, disproved by the following experimental results. Uv spectral analysis of the glycoside fraction, which contains genistein as its aglycone, did not show a bathochromic shift of band II after addition of NaOAc, but such a shift was found after acid hydrolysis, indicating that the OH group at C₇ provides a glycosidic linkage (Mabry *et al.*, 1970). Quantitative determination of the components of an acid hydrolysate of this fraction gave a 1:1.4 weight ratio between sugar and aglycone, corresponding to a 1:1 molar ratio, i.e. this fraction contains solely genistin. The conversion of

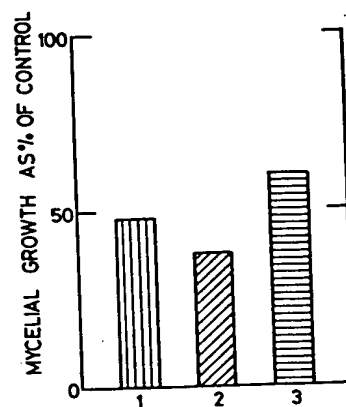


Figure 4. Effect of a mixture of isoflavone aglycones (0.1%) in growth medium on growth of *R. solani*: (1) native aglycones; (2) methylated aglycones (both measured by area of mycelium); (3) native aglycones (measured by dry weight).

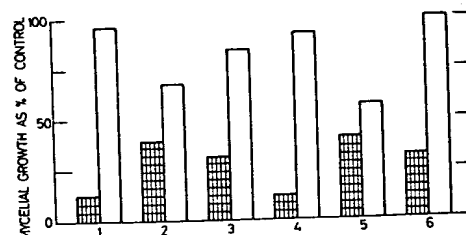


Figure 5. Effect of 0.1% of mixtures of isoflavone aglycones (▨) or isoflavone glycosides (□) in growth medium on mycelial growth of various fungi: (1) *Trichoderma lignorum*; (2) *Rhizoctonia solani*; (3) *Fusarium oxysporum*; (4) *Pythium* spp; (5) *Sclerotium rolfsii*; (6) *Rhizopus* spp.

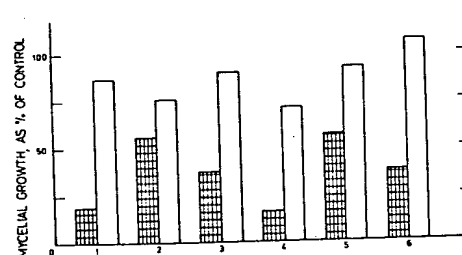


Figure 6. Effect of 0.005% of mixtures of isoflavone aglycones (▨) or isoflavone glycosides (□) in growth medium on mycelial growth of various fungi; see caption to Figure 5 for 1–6.

the glycoside of daidzein to formononetin (7-hydroxy-4'-methoxyisoflavone) or of the glycoside of glycitein to afrormosin (7-hydroxy-4',6-dimethoxyisoflavone) could only have occurred when the sugar moiety was linked exclusively to the OH group at C₇ (Naim *et al.*, 1973). Thus it could be concluded that the carbohydrate moiety of each glycoside is composed only of one glucose residue, which is invariably linked to C₇ of the aglycone.

The uv spectrum of glycitein 7-*O*- β -glucoside had the following absorption: (MeOH) λ_{max} 318, 259, 228 (sh); (MeOH + NaOMe) 322 (sh), 272, 223; (MeOH + AlCl₃) 312, 264, 228 (sh); (MeOH + AlCl₃-HCl) 312, 263, 228 (sh); (MeOH + NaOAc) 316, 260, 229; (MeOH + NaOAc-H₃BO₃) 318, 260, 230.

Quantitative Determination of Isoflavones in Soybeans. The quantitative determination of isoflavones was performed in the ether extract of soybean meal, in the methanol extract of soybean meal, and in the acid hydrolysate of the residual soybean flour after etheric and methanolic extractions. These three extracts were subject-

Table I. Characterization of Soybean Meal Isoflavone Aglycones by Tlc and Glc^a

Sample	Glc, 0.75% SE-30 on Gas Chrom Q	Tlc with solvent			Color appearance upon irradi. at 360 nm	R _f with Cloc re:
		B	A	C		
Genistein	1.65	1	1	1	Deep purple	
Daidzein	1.40	0.65	0.76	0.54	Fluorescent green	
Glycitein	2.3	0.53	0.89	0.32	Fluorescent light blue	

^a R_f genistein values were used in tlc and T_R formononetin values were used in glc of soybean isoflavones.

Table II. Content of the Different Isoflavones Present in the Ether Extract of Whole Soybean Meal, in the Methanol Extract of Soybean Meal, and in the Acid Hydrolysate of the Residual Soybean Flour after Eth and Methanolic Extractions

Fraction	mg/100 g of soybeans					Glycit 7-O- glucos
	Genistein	Genistin	Daidzein	Daidzin	Glycitein	
Ether	0.2		0.3		0.01	
Methanol	1.2	157.2	0.3	56.1	0.1	32.2
Residue		7.2		2.0		1.7
Total	1.4	164.4	0.6	58.1	0.11	33.8

ed to polyamide column chromatography of the nonche-
lated glycosides. Glc analyses of the emerging peaks
showed that the peaks corresponding to the glycosides
which resulted from the methanolic extract of soybean
were free from interfering substances (Figures 3a and b).
After hydrolysis the isoflavones could be determined by
glc according to the gas chromatographic procedure of
Lindner (1967). The transfer through the polyamide col-
umn and the hydrolytic treatment, which resulted in loss-
es of 15 and 5%, respectively, were taken into account.

Since accompanying substances interfered with the
quantitative gas chromatographic determination of free
isoflavones in the etheric and methanolic extracts of soy-
bean meal, their amount (traces) was determined by the
method of Beck (1964). As shown in Table II, practically
all the isoflavones in soybeans are present as glycosides,
and the free isoflavones amount to less than 1% of the
total. About 4% of the glycosides were still found in the
residual soybean flour, obtained after etheric and metha-
nolic extractions. The total percentage of isoflavones in
soybeans is about 0.25%. The amount of genistin is con-
siderably higher than of those of the two other isoflavones.

The finding that soybeans contain isoflavone aglycones
which are soluble in ether raised the question of their pos-
sible presence in commercial soybean oil. Samples of 200
ml of commercial soybean oil (Shemen Co., Haifa, Israel)
were subjected to the procedure of isolation of free isofla-
vones, including column chromatography on polyamide.
No free isoflavone aglycones could be identified in the
various fractions by tlc and glc. It may therefore be con-
cluded that no detectable isoflavone aglycones are present
in commercial soybean oil, probably since the commercial
process uses petroleum ether for extraction.

Effect of Soybean Isoflavones on Growth of Fungi.
Isoflavones exert pronounced fungistatic activity, as mea-
sured by the area of the mycelium as well as by the dry
matter content (Figures 4-6).

It is remarkable that a significant fungistatic activity
was noted at concentrations as low as 0.005% of free iso-
flavones (Figure 5) and it was not exceeded when the con-
centration was raised to 0.1% (Figure 6). The fungistatic
potency of isoflavone glycosides was, however, very limit-
ed. A direct relationship was found between the extent of
growth depression caused by isoflavone glycosides and the
amount of free isoflavones released into the growth medi-

um (unpublished results). It is assumed that fungal
cellular glycosidases enhance the fungistatic activi-
ty of isoflavone glycosides by releasing free isoflavones.

Experiments with *R. solani* showed that the dif-
ferent free isoflavones possess similar fungistatic potency.
also found that methylation of hydroxyl groups increas-
ed significantly ($P < 0.05$) the fungistatic activity (Fig-
ure 5). This is probably by making the isoflavones more lipophilic,
thus facilitating their passage through the cytoplasmic
membrane. These findings are consistent with pre-
vious results (Figures 5, 6), which showed a greater fungi-
cidal activity of free isoflavones as compared to that of the
glycosides. Increase of polarity of the inhibiting agent
tends to reduce its activity.

In order to ascertain that isoflavones do indeed
penetrate into the cytoplasm and can be translocated in
the fungus, the "dish technique" of Schutte (Figure 1)
was adopted. By using this method diffusion of the test
material through the growth medium is prevented. If the
translocated isoflavones are found in mycelial parts, which
have no direct contact with the growth medium contain-
ing isoflavones, this can be taken as proof for their trans-
location by the fungus. Such translocation could be shown
in the case of soybean isoflavones. It made no difference
whether the mature point of the mycelium (the inoculum)
or the younger one (the apex) was brought into contact
with agar containing isoflavones; they could be identified
by tlc in every part of the mycelium.

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Physical-Chemical Characteristics and Heavy Metal Content of Corn Grown on Sludge-Treated Strip-Mine Soil

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Corn was grown on strip-mine soil where anaerobically digested liquid sludge had been applied at a rate of 25 tons of sludge solids per acre. An adjacent plot of soil received no sludge. Corn grain grown on untreated strip-mine soil was characterized as immature and kernel size varied from small to intermediate, with about 20% of the kernels being diseased. In contrast, sludge-grown corn was well developed and corn yield increased fourfold over the untreated corn. Furthermore, a

significant protein enhancement of 2.5 percentage points was also realized. Concentrations of seven heavy metals (Zn, Mn, Cu, Pb, Cr, Cd, Hg) increased in grain, cobs, and husks in that order. For corn grain grown on untreated and sludge-treated soils, essentially no significant differences were found in heavy metal content when compared to 11 other corn varieties grown normally. Heavy metal contents of both soil and sludge samples were also determined.

A preliminary investigation was initiated to study corn grown under unique conditions—on strip-mine land treated with anaerobically digested liquid sludge. The Metropolitan Sanitary District of Greater Chicago has instituted a project in Fulton County in Central Illinois encompassing two major objectives: (a) disposing of anaerobically digested sludge and (b) upgrading organically deficient strip mine soil to the point where it can be productive agriculturally. Corn, a product of major commercial importance in this area, was chosen as the demonstration crop to study the effects of land application of sludge under specific controlled conditions.

A commercial yellow seed corn was planted in a tilled field of strip-mine soil. For comparative purposes, one plot was treated with sludge representing an equivalent of 25 tons of solids per acre, and an adjacent plot was not treated with sludge. On October 28, 1971, corn samples representing sludge-treated and untreated conditions were collected.

We measured the major physical and chemical characteristics of the whole kernel corn produced and grown under these two conditions. In addition, we determined the heavy metal content for parts of the corn plant that were contiguous with the whole kernel to establish the uptake of these metals. We wanted to answer the important question—are heavy metals, which are present in the sludge in very small quantities, translocated by way of the soil to the various parts of the corn plant, particularly the

edible parts? The metals studied included zinc, manganese, copper, lead, chromium, cadmium, and mercury.

Previously, Braids *et al.* (1970) studied the effect on crop yields of Reed Canary grass and sorghum grain but where digested sludge was applied to lysimeters. They also determined the elemental uptake of manganese, iron, copper, and zinc by corn grain and leaves.

MATERIALS AND METHODS

Sample Collection and Preparation. In each plot (untreated and sludge-treated), corn ears with husks intact were removed from the plants in the field and placed in large plastic-lined paper bags. Each bag contained ears representing many plants, and several bags were filled from each plot. The sealed bags were placed in cold storage at -29° until the samples were to be examined.

For analytical work, a composite whole grain corn sample representing each plot was prepared. For representative corn ear selection, all the bags were removed from cold storage and the contents were allowed to come to ambient laboratory temperature. All the ears of corn were separated into three relative sizes: large, medium, and small. An equal number of ears of each of the three sizes was then taken to start the formation of the composite grain sample. To avoid cross-contamination between untreated and sludge-treated samples, work on each lot was done on separate days.

The husks were removed and collectively gathered in a large plastic bag. The ears with the kernels intact were weighed and recorded. The kernels from each ear were then removed by hand and counted and both kernels and cob were weighed separately. To form a composite grain sample, all the kernels from each succeeding ear of corn were combined cumulatively until the total exceeded 10,000 kernels. The resultant composite samples required

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GENISTEIN INHIBITION OF THE GROWTH OF HUMAN BREAST CANCER CELLS: INDEPENDENCE FROM ESTROGEN RECEPTORS AND THE MULTI-DRUG RESISTANCE GENE

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The effect of isoflavones on the growth of the human breast carcinoma cell lines, MDA-468 (estrogen receptor negative), and MCF-7 and MCF-7-D-40 (estrogen receptor positive), has been examined. Genistein is a potent inhibitor of the growth of each cell line (IC_{50} values from 6.5 to 12.0 μ g/ml), whereas biochanin A and daidzein are weaker growth inhibitors (IC_{50} values from 20 to 34 μ g/ml). The isoflavone β -glucosides, genistin and daidzin, have little effect on growth (IC_{50} values >100 μ g/ml). The presence of the estrogen receptor is not required for the isoflavones to inhibit tumor cell growth (MDA-468 vs MCF-7 cells). In addition, the effects of genistein and biochanin A are not attenuated by overexpression of the multi-drug resistance gene product (MCF-7-D40 vs MCF-7 cells). © 1991 Academic Press, Inc.

Rats consuming a soy-based diet develop a lower number of mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]anthracene than rats on isonitrogenous and isocaloric diets without soy (1). We have speculated (1) that the aglucones of the isoflavones in soy, genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-

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Abbreviations: ER, estrogen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MDR, multi-drug resistance; TPK, tyrosine protein kinase; EGF-R, epidermal growth factor receptor; HPLC, high performance liquid chromatography; gp 170, a 170,000 Da glycoprotein, a product of the multidrug resistance gene.

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dihydroxyisoflavone), may have properties similar to the antiestrogen drug tamoxifen, which competes with estrogen for occupancy of the estrogen receptor (ER), thereby inhibiting the metastatic growth of breast cancer. On the other hand, it has been shown that isoflavones, particularly genistein, are potent inhibitors of the tyrosine protein kinase (TPK) activity of growth factor receptors, such as epidermal growth factor receptor (EGF-R) (2), and several oncogenes which may be associated with tumor cell growth and tumor recurrence, such as Ha-ras (3) and pp56^{lck}, a src-family kinase (4). Since many recurrent breast cancers are ER-independent, a drug or dietary agent that inhibits the growth of both ER⁺ and ER⁻ tumors would be of great interest.

In addition, it is important to determine whether such compounds are substrates of the multi-drug resistance (MDR) gene product, P-glycoprotein. This 170 KDa cell membrane protein (gp 170) confers resistance to a wide range of chemotherapeutic agents by acting as a drug efflux pump, thereby reducing the concentration of the drug in the cytoplasm of the tumor cell (5).

In this study, we have examined whether: (1) soy isoflavones inhibit the growth of human breast cancer cells in culture, (2) whether inhibition is dependent on the expression of the ER and (3) whether inhibition is attenuated by expression of gp 170.

MATERIALS AND METHODS

Materials: Soy molasses was a gift of the Archer Daniels Midland Co. (Decatur, IL). Fetal bovine serum and antibiotics were obtained from Gibco (Gaithersburg, MD). Tissue culture supplies were from Costar (Charlotte, NC). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), doxorubicin, α - and β -glucosidases and biochanin A (4'-methoxygenistein) were from Sigma Chem. Co. (St. Louis, MO). Microtiter plates, Sephadex G-25 and CNBr-activated Sepharose were purchased from Pharmacia (Piscataway, NJ). Aquapore C₈ columns were from Brownlee Labs (Santa Clara, CA).

Cell Culture: MCF-7 and MCF-7 D-40, and MDA-468 human breast cancer cell lines were gifts of Dr. William Dalton (University of Arizona) and Dr. Jeff Kudlow (Division of Endocrinology, University of Alabama at Birmingham), respectively. MCF-7 and MCF-7 D-40 cells were maintained in RPMI 1640 medium supplemented with 7% (v/v) fetal-bovine serum and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin); MCF-7-D-40 cells also received 10⁻⁸ M doxorubicin to maintain the MDR phenotype; MDA-468 cells were maintained on Dulbecco's Modified Eagles medium low glucose, with 10% (v/v) fetal bovine serum and antibiotics (as above). Cells were cultured as

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Isoflavone Preparation: Genistin and daidzin were isolated from soy molasses by fractional crystallization (6) and by adsorption chromatography (7), respectively. Their aglucones, genistein and daidzein, were prepared by hydrolysis in methanol: 1 M HCl (1:1 v/v).

Sample Preparation: Isoflavone samples were prepared from 10, 5, or 2.5 mg/ml stock solutions in DMSO. Aliquots were then taken to prepare the various samples (final concentrations from 1 to 100 μ g/ml). DMSO was added as necessary to give a final DMSO concentration of 1% (v/v) in each well.

HPLC Analysis: The purity of the stock solutions and the stability of the isoflavones in the tissue culture media during incubation with the cells were determined by reversed-phase HPLC on a 30 x 0.45 cm Aquapore C₈ column using gradient elution with a mobile phase consisting of 0-45% acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. Eluting substances were detected by their absorbance at 262 nm.

Viability Assay: Cytotoxicity of the isoflavones was determined by a modification of the MTT assay (8), which is based on the reduction of MTT by the mitochondrial dehydrogenases of viable cells. Cells were plated into 96-well tissue culture clusters at densities of 2×10^3 cells/well (MCF-7), 10^4 cells/well (MCF-7-D40) and 2.5×10^3 cells/well (MDA-468) in 198 μ l of media (optimal numbers of cells for each well were previously determined by ³H-thymidine uptake). After plating, the cells were allowed to attach for 2 days. Isoflavones were then added (2 μ l volumes as described) and incubation continued for 4 days; control wells received 2 μ l DMSO. After 4 days, 50 μ l of 2 mg/ml MTT was added to each well and the plates incubated for 4 h at 37° C. Media and unreacted MTT were then removed by gentle aspiration. One row of cells had the media removed for HPLC analysis. DMSO (100 μ l) was added to each well and the plates were gently shaken for 5 min at room temperature. The optical density at 540 nm was immediately determined using a MAXLINE plate reader (Molecular Devices, Menlo Park, CA). Absorbance at 690 nm was also measured to compensate for interfering effects of cell debris and the plate itself. The percent survival was determined by comparing the absorbance for treated cells to that obtained for control cells. Each experiment consisted of 3 plates, and the results given are the mean and standard error of three separate experiments.

RESULTS AND DISCUSSION

Genistein was a potent growth inhibitor in both MCF-7 cells (IC₅₀ 10.5 μ g/ml) and MDA-468 cells (IC₅₀ 6.5 μ g/ml) (Fig. 1A). Biochanin A had weaker inhibitory effects on the growth of MCF-7 and MDA-468 cells (IC₅₀ values of 22 μ g/ml and 30 μ g/ml, respectively) (Fig. 1B). Daidzein, also had weak effects on cell growth, with IC₅₀ values of 28 μ g/ml for MCF-7 cells and 34 μ g/ml for MDA-468 cells (Fig. 1C). The isoflavone β -glucosides, genistin and daidzin, were not effective

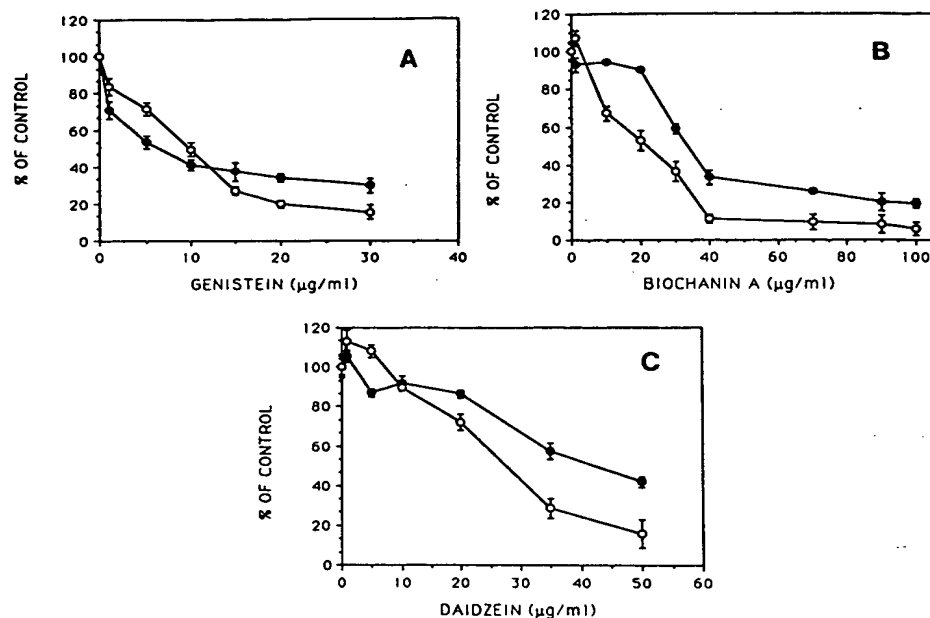


Figure 1. Inhibition of growth relative to controls of MCF-7 (O) and MDA-468 (●) cells by the isoflavones genistein (panel A), biochanin A (panel B) and daidzein (panel C).

in inhibiting cell proliferation, with IC_{50} values above $100 \mu\text{g/ml}$ (Fig. 2A and B). The weak growth inhibition observed at higher concentrations of genistin in MDA-468 cells appeared to be due to tumor-cell induced hydrolysis of genistin to genistein (data not shown). This hydrolysis did not occur in MCF-7 cells.

There was no significant difference in the potency of growth inhibition of MDA-468 and MCF-7 cells by each isoflavone. These data suggest that the

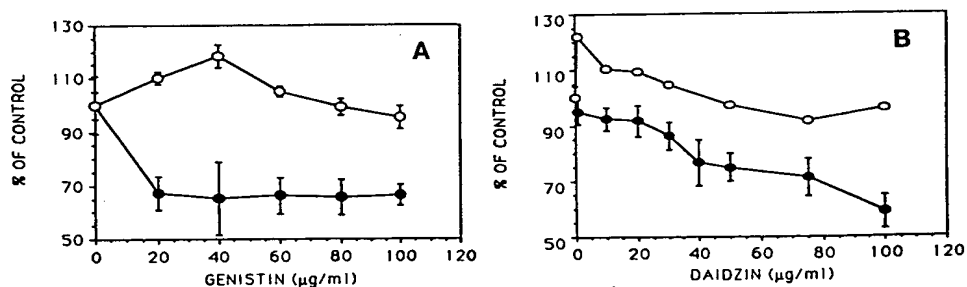
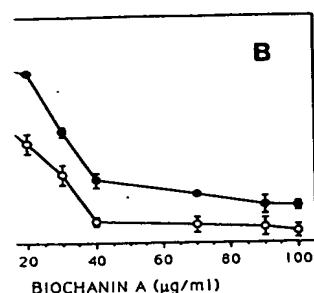
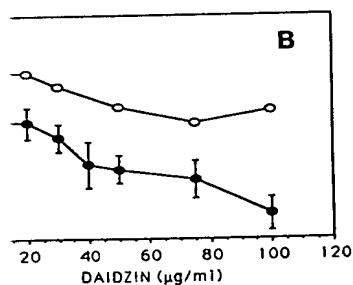


Figure 2. Inhibition of growth relative to controls of MCF-7 (O) and MDA-468 (●) cells by the isoflavones genistin (panel A) and daidzin (panel B).



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isoflavones can act via an ER-independent pathway. This does not, however, rule out the involvement of ER in isoflavone action in ER⁺ cells. Recent evidence has shown that increased phosphorylation of the estrogen and progesterone receptors can alter the activity of these receptors (9,10). The isoflavones could, therefore, in part, exert their effect by interfering with their phosphorylation state.

The precise mechanism of action of genistein, and of isoflavones in general on tumor cell proliferation is at present unknown. The effect of genistein is however, non-specific; although the growth of *ras*-transformed NIH-3T3 cell was inhibited by genistein, the growth of non-transformed cells at the same genistein concentration was unaffected (11). Genistein inhibits the intrinsic TPK activity of many growth factor receptors, including EGF-R (2,3) and platelet-derived growth factor receptor (12). The isoflavones could also inhibit targets downstream of the activated receptor such as phospholipase C- γ , phosphatidylinositol kinases, or MAP kinase, all of which show increased tyrosine phosphorylation in response to EGF treatment (13,14,15,16). In support of this view, the isoflavone psi-tectorigenin (8-methoxygenistein) has been shown to inhibit cellular phosphatidylinositol turnover without inhibiting EGF-R TPK activity in A431 fibroblasts (17). Also, genistein can cause cytostatic effects on cell growth without inhibiting the EGF-R TPK activity in NIH-3T3 cells, possibly due to its preferential inhibition of ribosomal S6 phosphorylation (18), which is thought to occur via MAP kinase (19). However, Ogawara et al. (3) found no close correlation between inhibition of EGF-R tyrosine kinase activity *in vitro* and the reduction in the growth of Ha-*ras* transformed NIH-3T3 cells.

An alternative mechanism for the action of isoflavones is their inhibition of DNA topoisomerases. Genistein has been shown to inhibit mammalian DNA topoisomerase II in L-1210 cells (12). Also, a chinese hamster ovary cell line with altered DNA topoisomerase activity has been isolated that is more resistant to genistein than the parental cell line (20).

A derivative of the MCF-7 cell line, MCF-7-D40, which overexpresses gp 1 is resistant to the potent anticancer drug doxorubicin (Fig. 3A) (6). However

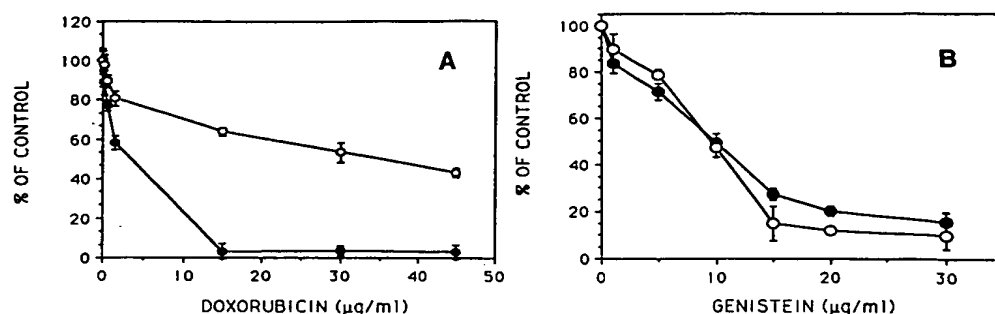


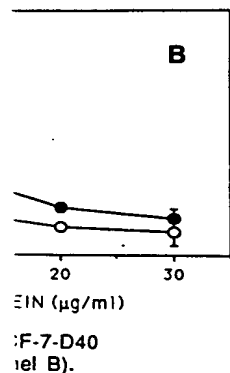
Figure 3. Inhibition of growth of MCF-7 wild type (●) and MCF-7-D40 (○) cells by doxorubicin (panel A) and genistein (panel B).

IC₅₀ for genistein was the same for the MCF-7 and MCF-7-D40 cell lines (Fig. 3B). In addition, the IC₅₀ for biochanin A was lower in the MCF-7-D40 cells than in the MCF-7 cells (data not shown). These results show that neither genistein nor biochanin A are adversely effected by overexpression of gp 170, and suggest that the isoflavones, in general, may be immune to the multidrug resistance phenomena. In support of this observation, Honma et al. have shown that genistein induces differentiation of a multi-drug resistant K562 (human myelogenous leukemia) cell line as effectively as in its parental cell line (21).

The data obtained in this study support the notion that the isoflavones genistein and daidzein are active anti-cancer agents in soy. Thus soy, a significant part of the diet of many Orientals, may be an important factor which accounts for the low rate of breast cancer in Oriental women (22).

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NEW ISOFLAVONOIDS AS INHIBITORS OF PORCINE 5-LIPOXYGENASE

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Abstract—The inhibitory activity of new isoflavonoids on 5-lipoxygenase of porcine leukocytes was investigated. Isoflavans (I) proved to be stronger inhibitors than isoflavones (II). The isoflavans containing *ortho*-hydroxy groups in ring A showed the lowest K_i values (0.8–50 μM). In comparison, isoflavans with *meta*-dihydroxy groups exhibited K_i values higher than 150 μM . The effect of commercial antioxidants was tested also on porcine 5-lipoxygenase. Butylated hydroxyanisole (K_i : 25 μM) and butylated hydroxytoluene (K_i : 55 μM) revealed moderate inhibitory activity, whereas L-ascorbic acid, L-ascorbyl palmitate, *dl*- α -tocopherol and *n*-propyl gallate showed weak inhibitory activities (K_i : 100–260 μM).

Isoflavonoids represent an important subclass of the flavonoids. The structure of isoflavonoids is based on a 3-phenylchroman skeleton, which biogenetically is derived by aryl migration from the 2-phenylchroman skeleton of the flavonoids [1]. While flavonoids are ubiquitous compounds, the isoflavonoids show a limited distribution in the plant kingdom. They are found mainly in the subfamily *Papilionoidae* of the Leguminosae (for review see Ref. 2).

Isoflavonoids are known to exhibit various biological properties, e.g. the insecticidal activity of rotenoids [2] and isoflavans [3], the anti-microbial, especially the anti-fungal, activity of the phytoalexins (pterocarpan, isoflavans and some isoflavones [2, 4]), an hypocholesterolemic effect and a triglyceride-lowering activity [5–7]. The isoflavonoids have many properties in common with the flavonoids, e.g. anti-cataract [8, 9] anti-inflammatory and anti-allergic activity [10–14]. Some biological activities are explained by special biochemical mechanisms. Thus, the anti-inflammatory and anti-allergic activity of flavonoids is in part due to inhibition of the enzymes involved in the arachidonic cascade [10–14].

One of the enzymes of the arachidonic acid cascade, the 5-lipoxygenase (5-LOX[†]) is the first enzyme in the biosynthetic pathway leading to LT. LTs are potent mediators, involved in immunoregulation and in various diseases, including inflammation, asthma and diverse allergic reactions. In neutrophils, stimulated with the Ionophore A 23187, the main products of arachidonic acid are 5-HETE and LTB₄ [15]. The same pattern of 5-LOX products is obtained with neutrophils derived from various species stimulated under the same conditions [16]. Previously, the HETEs were considered to be predominantly inactivation products of HPETEs

without biological importance. New investigations have shown that HETEs modulate basic biological functions such as enzyme regulation, hormone secretion, ion transport and immune mechanisms. They are involved in pathological processes including various inflammatory diseases, arteriosclerosis and ischemia (for review see Ref. 17).

Due to the participation of LT and HETEs in various diseases, we evaluate in this study the inhibitory effect of different new synthetic isoflavonoids on porcine 5-LOX *in vitro*. We also compare the effect of isoflavonoids on porcine 5-LOX with that of commercial food antioxidants.

MATERIALS AND METHODS

Materials

All isoflavonoids have been synthesized at the Institute for Physiological Chemistry of the University of Bonn [18]. The antioxidants *dl*- α -tocopherol, BHT, BHA and L-ascorbic acid were purchased from Merck (Darmstadt, Germany), *n*-propyl gallate from Sigma (Munich, Germany) and ascorbyl palmitate from Serva Feinbiochemica (Heidelberg, Germany). Dextran T-500 for cell sedimentation was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Arachidonic acid (Merck) was purified by silicic acid column chromatography prior to use. The Ionophore A 23187 and PGB₂, which served as internal standard, were obtained from Sigma. ETYA was supplied by Hoffmann-La-Roche (Basle, Switzerland). All salts, organic solvents, thin layer silicic acid plates and Trypan blue were obtained from Merck. All chemicals used were of reagent grade. The solvents for HPLC were dried, distilled and filtered.

5-LOX assay

Preparation of leukocyte suspension. Porcine peripheral blood leukocytes were prepared according to the method of Kuhl *et al.* [10]. Porcine blood (1.5 L) was decoagulated with 100 mL Hank's buffer solution containing 3.8% sodium citrate, 6 U of heparin/mL of blood and passed through a Dextran

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† Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; LOX, lipoxygenase; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; PG, prostaglandin; ETYA, 5,8,11,14-eicosatetraenoic acid.

(6%) gradient. After sedimentation at 4° for 60 min, the supernatant was centrifuged at 500 g for 12 min. The pellet was resuspended in Hank's buffer containing 0.38% sodium citrate. The centrifugation was repeated. Residual erythrocytes were lysed after 5 min incubation at 25° with Tris buffer (17 mM, pH 7.2) containing 0.17% ammonium chloride. After centrifugation at 400 g for 6 min and resuspension of the cells in Hank's buffer containing 0.38% sodium citrate, the solution was centrifuged again at 400 g for 6 min. The cell pellet was resuspended in phosphate-buffered (25 mM, pH 7.4) isotonic saline at 4×10^7 cells/mL. The viability of the cells (higher than 90%) was checked by Trypan blue exclusion.

Incubation conditions for porcine leukocytes. Leukocyte suspension (10 mL) was preincubated for 3 min at room temperature in the presence of different concentrations (0.5–200 μ M) of isoflavonoids or usual food antioxidants dissolved in ethanol or dimethyl sulfoxide. An equivalent suspension with solvent (ethanol or dimethyl sulfoxide) but lacking test substance served as control. The solvent content did not exceed 1%, to avoid an influence on 5-LOX activity [19]. During the assay for 5-LOX activity, nearly complete inhibition of 12-LOX is achieved by addition of 10 μ M ETYA [11] to the reaction mixture, as the latter enzyme is the predominant LOX of porcine leukocytes [20].

The 5-LOX reaction was started by adding the following substances to the leukocyte suspension: CaCl_2 (2 mM), Ionophore A 23187 (10 μ M), ETYA (10 μ M) and the substrate arachidonic acid (100 μ M). After incubating the cells for 5 min at 37°, the enzymatic reactions were stopped by adding 1.5 mL of formic acid (1%). After addition of PGB_2 (2 μ g) as an internal standard, the samples were extracted immediately with chloroform/methanol (1:1, v/v; 2×15 mL), evaporated and stored under nitrogen at -18° prior to HPLC analysis.

HPLC analysis. Analytical HPLC was performed according to Kuhl *et al.* [10] with slight modifications. A prepacked column (Hibar RT, 250 \times 4 mm, Lichrosorb 60, 7 μ m, Merck) and a precolumn (RCSS Silica T 61031) from Waters, Millipore (Eschborn, Germany) were employed (instrument: S 101, Siemens; pump: DMR-AE-10.4, Orlita; Injectorsystem: U6K, Waters).

The compounds were eluted using first *n*-hexane/2-propanol/methanol/acetic acid (972/18/9/1 by vol.) containing 0.06% water. After 9.5 min the gradient elution was started. The 2-propanol content was raised during a linear gradient up to 3 vol.% in 12 min (972/30/9/1). The flow rate was 3.5 mL/min at 22°. The elution was monitored spectrophotometrically at 235 nm (0–9.5 min) and at 280 nm (9.5–24 min). 5-HETE and LTB_4 were quantified by comparing their peak areas with that of PGB_2 (internal standard). The extinction coefficients used for 5-HETE, LTB_4 and PGB_2 were $\epsilon = 30,500$, 39,500 and 26,800 $\text{L mol}^{-1} \text{mm}^{-1}$, respectively.

RESULTS

Inhibition of porcine 5-LOX by isoflavonoids

Arachidonic acid incubated with porcine leuko-

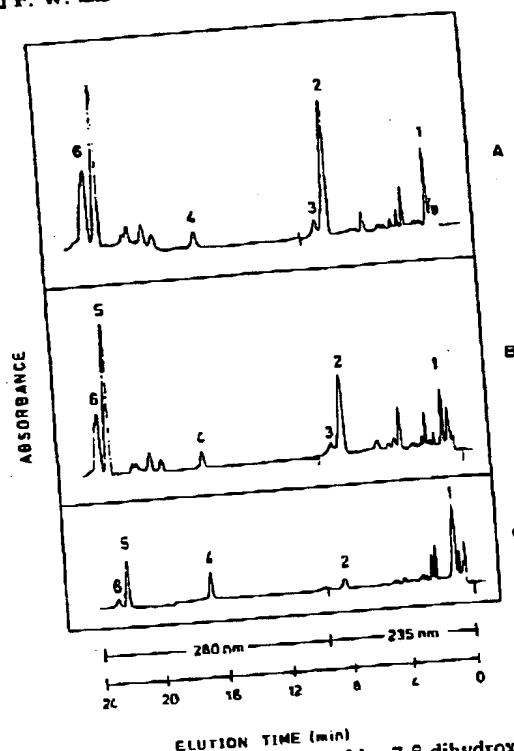
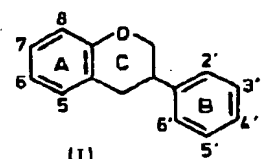


Fig. 1. Inhibition of porcine 5-LOX by 7,8-dihydroxy-4'-methoxyisoflavan (6). HPLC chromatograms of the products formed during a 5-min incubation of porcine peripheral blood leukocytes ($10 \text{ mL}/4 \times 10^7$ cells/mL) with arachidonic acid (100 μ M), CaCl_2 (2 mM), Ionophore A 23187 (10 μ M) and ETYA (10 μ M). (A) Control (without test substance), (B) with 10 μ M 7,8-dihydroxy-4'-methoxyisoflavan (6), (C) with 50 μ M 7,8-dihydroxy-4'-methoxyisoflavan (6). Signal attenuation was three times higher at 235 nm than at 280 nm. Peaks: 1, arachidonic acid; 2, 5-HETE; 3, 5-HPETE; 4, PGB_2 ; 5, LTB_4 ; 6, stereoisomers of LTB_4 .

cytes in the presence of Ionophore A 23187, CaCl_2 and ETYA is converted predominantly to 5-HETE and LTB_4 . Figure 1A shows a typical HPLC chromatogram of an incubation performed under the conditions described in Materials and Methods. The effect of various concentrations of 7,8-dihydroxy-4'-methoxyisoflavan (6) on 5-LOX-activity is shown in Fig. 1B and C. The formation of the 5-LOX products 5-HETE and LTB_4 was suppressed by 7,8-dihydroxy-4'-methoxyisoflavan (6) in a dose-dependent manner. The inhibition of 5-LOX by isoflavonoids is expressed as the percentage of activity related to the control value measured without inhibitor. Plotting of $1/(\% \text{ activity})$ vs $[I]$ was carried out to evaluate the inhibition constant K_i . The substitution patterns of isoflavans (Table 1) and corresponding K_i values. K_i values of most isoflavonoids tested ranged from 0.1–100 μ M.

Structure-activity studies showed that the isoflavans inhibited porcine 5-LOX more effectively than the corresponding isoflavones [see compounds (2) and (3), (13) and (14), (18) and (17)]. Looking

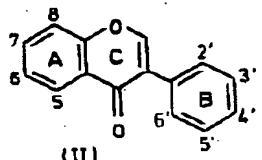
Isoflavonoid inhibition of porcine 5-LOX

Table 1. K_i values of porcine 5-LOX inhibition by various isoflavans


(I)

Compound	5	6	7	Substitution 8	3'	4'	K_i (μ M)
(1)	H	OH	OH	H	H	OH	37
(2)	H	H	OH	OH	H	OH	0.8
(4)	H	O-Ace	O-Ace	H	H	OCH ₃	15.5
(5)	H	OH	OH	H	H	OCH ₃	2.5
(6)	H	H	OH	OH	H	OCH ₃	21
(7)	H	OH	OH	H	H	CH ₃	53
(8)	H	H	OH	OH	H	CH ₃	7.5
(9)	OH	H	OH	H	H	CH ₃	218
(10)	H	OH	OH	H	OCH ₃	OCH ₃	7
(11)	H	H	OH	OH	OCH ₃	OCH ₃	24
(12)	H	H	OH	CH ₃	OCH ₃	OCH ₃	70
(13)	H	OH	OH	H	O-CH ₂ -O	OCH ₃	28
(18)	H	H	O-CH ₂ -O	H	H	OCH ₃	0.6
(22)	H	H	OH	OH	H	Cl	9.5
(23)	H	H	OH	CH ₃	H	Cl	36
(25)*	OH	H	OH	H	H	OH	168

* See Ref. 11.

Table 2. K_i values of porcine 5-LOX inhibition by various isoflavones


(II)

Compound	5	6	7	Substitution 8	3'	4'	K_i (μ M)
(3)	H	H	OH	OH	H	OH	19.5
(14)	H	OH	OH	H	H	O-CH ₂ -O	103
(15)	H	H	OH	OH	H	O-CH ₂ -O	23.5
(16)	H	H	O-CH ₂ -O	H	OCH ₃	OCH ₃	250
(17)	H	H	O-CH ₂ -O	H	H	OCH ₃	60
(19)	H	OH	OH	H	F	H	16.5
(20)	H	H	OH	OH	F	H	21
(21)	H	H	OH	OH	CF ₃	H	16
(24)	H	H	OH	OH	H	NO ₂	91

for an influence of the substituents in ring B, it is obvious that there is no specific relation to 5-LOX inhibition. Among the tested isoflavans and isoflavones neither size, position nor different charge of the substituents was decisive for inhibition strength (see compounds (3), (6), (8), (11), (20), (21), (22)). Comparing the effects of 6,7-dihydroxyisoflavonoids and of the 7,8-dihydroxyisoflavonoid

isomers on porcine 5-LOX, there was no structure-activity relationship [see compounds (1) and (2), (5) and (6), (10) and (11), (19) and (20)]. In contrast, structure-activity relationships are obvious comparing *ortho*-hydroxy- and *meta*-hydroxy-substituted compounds in ring A. Among the 4'-methylisoflavans, the *ortho*-hydroxy isoflavans (7) and (8) are significantly strong r inhibitors of porcine 5-

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Table 3. Inhibition of porcine 5-LOX by commercial food antioxidants (K_i values)

Compound	K_i (μ M)
BHA	25
BHT	55
<i>n</i> -Propyl gallate	124
<i>dl</i> - α -Tocopherol	259
L-Ascorbic acid	100
L-Ascorbyl palmitate	239

LOX than the respective *meta*-hydroxy isoflavan (9). A similar result was obtained in the corresponding series of 4'-hydroxyisoflavans [see compounds (1), (2) and (25)]. Comparing 7,8-dihydroxyisoflavans with the 7-hydroxy-8-methylisoflavans, the *ortho*-hydroxy substituted compounds are as expected more effective 5-LOX inhibitors [see compounds (11) and (12), (22) and (23)]. Surprisingly some isoflavans, which lack free *ortho*-dihydroxy-substituents in ring A show a marked 5-LOX inhibition. These compounds are 6,7-methylenedioxy-4'-methoxyisoflavan [(18), K_i : 0.6 μ M] and 6,7-diacetyl-4'-methoxyisoflavan [(4), K_i : 15.5 μ M].

Inhibition of porcine 5-LOX by food antioxidants

In Table 3 the K_i values for inhibition of porcine 5-LOX by commercially available food antioxidants are summarized. Whereas BHA (K_i : 25 μ M) and BHT (K_i : 55 μ M) are moderate inhibitors, L-ascorbic acid, L-ascorbyl palmitate, α -tocopherol and *n*-propyl gallate are less effective inhibitors (K_i : 100–260 μ M) than most isoflavans.

DISCUSSION

Inhibition of porcine 5-LOX by isoflavonoids and food antioxidants

The different isoflavonoids tested in this study inhibited the porcine polymorphonuclear leukocytes 5-LOX in a concentration range of 0.1–100 μ M (K_i and IC_{50} values). The same concentration range was obtained with most flavonoids containing also hydroxy- and methoxy-substituents [21–25]. The

absolute inhibition values found for various compounds in the literature, however, differ depending on the type of LOX, the enzyme source, method of enzyme isolation and the assay conditions. This fact is illustrated in Table 4 for quercetin, which may be designated as reference flavonoid. Therefore comparison of different studies should be undertaken with certain reservations.

Other compounds tested as inhibitors of porcine 5-LOX are: (*E/Z*)-Ajoene (IC_{50} : 1.6 μ M), a garlic constituent [30]; nordihydroguaiaretic acid (IC_{50} : 1.5 μ M); caffeic acid (IC_{50} : 46 μ M); *p*-coumaric acid (IC_{50} : 2.5 μ M) and wedelolactone (IC_{50} : 2.5 μ M), a coumestane derivative [19]. These compounds inhibited the 5-LOX also to the same extent as the isoflavonoids tested in this study.

The structure-activity relationship of the isoflavonoids concerning 5-LOX-inhibition (Tables 1 and 2) revealed the following conclusions: (i) isoflavans were found to be more effective inhibitors than their corresponding isoflavones. An explanation may be the change in conformation of ring C after hydration of the isoflavones to isoflavans and the interruption of the fully conjugated system. (ii) *ortho*-Dihydroxy-substituted isoflavans (1), (2), (7) and (8) inhibited the 5-LOX at lower concentrations than the respective *meta*-dihydroxy-substituted isoflavans (9) and (33). The 7,8-dihydroxyisoflavans also proved to be stronger 5-LOX inhibitors than the 7-hydroxy-8-methylisoflavans (12) and (23).

Other authors [31] found that phenolic *ortho*-dihydroxy-compounds, including caffeic acid and the flavonoids taxifolin (3,5,7,3',4'-pentahydroxyflavonoid), luteolin (5,7,3',4'-tetrahydroxyflavon) and vanon (3,5,7,3',4'-pentahydroxyflavon), clearly had strong radical-scavenging activities, whereas monohydroxylated and *para*-dihydroxylated compounds proved to be only moderate to weak radical scavengers. Compounds lacking a free hydroxy-group in the molecule scarcely influenced radical scavenging. Some isoflavonoids examined in our study may also trap radicals. During this reaction isoflavonoids are oxidized to *ortho*- and/or *para*-benzoquinones as shown here for 6,7- and 7,8-dihydroxyisoflavans in Fig 2a and b.

Oxygen radicals, which are probably involved in inflammatory and cancerogenic processes, are generated as by-products during arachidonic acid metabolism via the cyclo-oxygenase and LOX

Table 4. Inhibition (IC_{50} values) of various LOXs by quercetin

Enzyme source	IC_{50} (μ M)	Literature
5-LOX	0.2	[26]
5-LOX	2.1	[22]
5-LOX	0.1–1	[23]
5-LOX	>1	[21]
5-LOX	0.8	[11]
5-LOX	\approx 125	[27]
5-LOX	4–5	[26]
12-LOX	2–3	[28]
13-LOX	>10	[29]
15-LOX		

Isoflavonoid inhibition of porcine 5-LOX

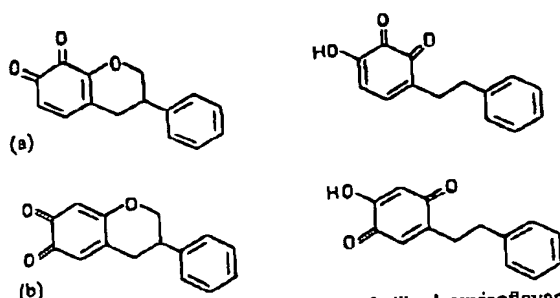


Fig. 2. (a) *ortho*-Benzoquinones of 7,8-dihydroxyisoflavan. (b) *ortho*- and *para*-Benzoquinones of 6,7-dihydroxyisoflavan.

pathway. It is known that fatty acid hydroperoxides formed in LOX reactions are necessary for LOX activity [32–35]. Therefore, trapping of these radicals by isoflavonoids may reduce the 5-LOX activity. But not all compounds with radical-scavenging properties are effective antioxidants.

LOXs are non-heme iron-containing enzymes [36, 37] existing in two different forms: a Fe^{3+} (ferric) and a Fe^{2+} (ferrous) form [38, 39]. Kemal *et al.* [34] showed that catechols reduced the catalytically active ferric of soybean LOX to the inactive ferrous form. The reduction of the Fe^{3+} LOX to the inactive Fe^{2+} form also may be performed by *ortho*-dihydroxyisoflavonoids, according to the redox cycle for LOX activation and inactivation proposed by Hatzelmann *et al.* [35]. Isoflavonoids may act as the H-atom donor and/or the radical scavenger in this cycle.

6,7-Diacetyl-4'-methoxyisoflavan (4) and 6,7-methylenedioxy-4'-methoxyisoflavan (18) which both lack the free hydroxy- group showed a strong inhibition of 5-LOX. The diacetyl- and the methylenedioxy- groups may be split off by cellular hydrolases to yield free hydroxy- groups.

The antioxidative activity of the isoflavonoids studied was examined in our laboratories [6, 7, 40]. Isoflavans, especially those containing *ortho*-dihydroxy- groups in ring A, inhibited strongly the auto-oxidation of vitamin E-free lard (this is for the most part in accordance with the structure-relationship found for 5-LOX inhibition). 6,7-Dihydroxyisoflavonoids from fermented soy oil were 10–20 times stronger antioxidants than vitamin E [41–43].

Comparing the effect of *dl*- α -tocopherol and the *ortho*-dihydroxyisoflavans on porcine 5-LOX, the isoflavonoids proved to be on average 150 times more active than the tocopherol. K_i values for *n*-propyl gallate, L-ascorbic acid and ascorbyl palmitate were also higher than those for the isoflavans. The inhibitory potency of BHA and BHT is comparable with the inhibition caused by isoflavonoids. However, the use of BHA and BHT as food additives is controversial [44, 45].

During the 5-LOX assay viability of the leukocytes (Trypan blue exclusion) in the presence of isoflavonoids was 80–105% of the control. In the presence of BHT the viability decreased to 37% of

the control value. Some isoflavonoids were tested on P 388 leukemia in mice and were found, up to 240 mg/kg test animal, not to exert any cytotoxic effect [46]. The bioavailability of a physiologically active compound tested *in vitro* is one of the factors determinative for a possible therapeutic use of such compounds. Few data are available on the metabolism and pharmacokinetics of isoflavonoids [9, 47] (for review see Ref. 48). These studies concerned distribution, storage and elimination of isoflavonoids. We have investigated the absorption of some isoflavonoids on isolated intestinal segments of the rat (unpublished data).

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3-Hydroxylation of 4'-Methoxyisoflavones by *Fusarium oxysporum* f. *lycopersici*

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Hydroxylation, Isoflavones, *Fusarium*, Metabolism

3-Hydroxylation of isoflavones by *Fusarium oxysporum* f. *lycopersici* mainly proceed with 4'-methoxy-7-hydroxyisoflavones; this reaction is used for quantitative conversion of ¹⁴C-labelled isoflavones.

Introduction

Due to their fungitoxic properties isoflavones and pterocarpan represent interesting substrates for fungal metabolism [1]. Among the various initial reactions of isoflavone metabolism by *Fusarium* (fungi [2-4]) 3-hydroxylation is recently found [3] with the isoflavones biochanin A (Fig. 2, 1a) and formononetin (1b) is of special interest. Hydroxylation adjacent to an existing methoxyl group has only rarely been found and warrants further investigations.

Using a strain of *Fusarium oxysporum* f. *lycopersici* we report some results on the substrate specificity of isoflavone 3-hydroxylation, the quantitative extent of this reaction and on its use for obtaining ¹⁴C-labelled isoflavones.

Results and Discussion

In standard incubation assays with mycelial preparations of *F. oxysporum* f. *lycopersici* isoflavone anions of (10^{-4} M) metabolism was quantitatively followed in aliquots by either TLC (SI) with subsequent scanning by case of ¹⁴C-labelled substrates, or by HPLC. Thus, the efficient conversion of 1a and 1b into pratensein (IIIa) and calycosin (IIIb), respectively, could be demonstrated. As shown in Fig. 1 biochanin A 3-hydroxylation proceeds much more rapidly than formononetin metabolism. Quantitative accumulation of pratensein is reached after approx. 13 h whereas maximum formation of IIIb requires some 28 h. Under our experimental conditions IIIa is appeared to be an endproduct whereas IIIa is slowly further degraded. Attempts to isolate any metabolites of IIIa have so far failed.

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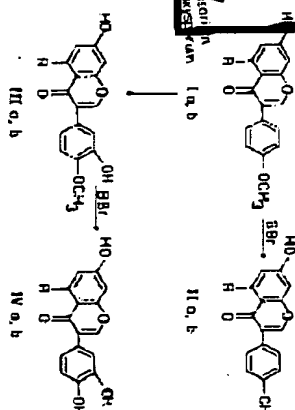


Fig. 2. The isoflavones biochanin A (1a) or formononetin (1b) may quantitatively be converted into a series of hydroxyderivatives by a combination of fungal (3-hydroxylation by *F. oxysporum* f. *lycopersici*) and chemical reactions. This scheme is used to prepare ¹⁴C-labelled isoflavones IIIa-IVb from ¹⁴C-1a and -1b.

1a R = -OH; Biochanin A
1b R = -H; Formononetin
IIa R = -OH; Genistein
IIb R = -H; Daidzein
IIIa R = -OH; Pratensein
IIIb R = -H; Calycosin
IVa R = -H; 7,3',4'-Trihydroxyisoflavone
IVb R = -H; 7,3',4'-Trihydroxyisoflavone

methoxyisoflavone (lexatin) were not 3-hydroxylated by *F. oxysporum* f. *lycopersici*.

The enzyme activity of this *Fusarium* strain for isoflavone 3-hydroxylation which appears to be rather specific for a 7-hydroxy-4'-methoxyisoflavone skeleton, did not readily respond as an inducible enzyme system. Preincubation of mycelial preparations with 1a or 1b for up to 27 h did not significantly shorten the lag phase of 3-hydroxylation nor increase the velocity of isoflavone metabolism.

The quantitative 3-hydroxylation of both biochanin A and formononetin (Fig. 1) has been used as one step in the preparation of ¹⁴C-labelled isoflavones with a 3',4'-disubstituted B-ring. [¹⁴C]biochanin A or formononetin accessible in rather large amounts by application of ¹⁴C-labelled acetate, phenylalanine or cinnamic acid to roots of chick pea plants (*Cicer arietinum* L.) [5] may readily be converted by *F. oxysporum* to pratensein or calycosin in 100 mg quantities. Subsequent O-demethylation to IIIa and IVb with BBr₃ also proceeds quantitatively [6]. As shown in Fig. 2 a combination of fungal and chemical reactions leads from 1a or 1b to a variety of other isoflavones (IIIa-IVb) which may thus be synthesized in position-specific labelled form in excellent yield.

Experimental

Fungus

Fusarium oxysporum Schlecht ex Fr. f. *lycopersici* (Sacc.) Syn. u. Hans. (Centraalbureau voor Schimmelcultures, CBS 163.30) was stored and grown as previously described [3].

Standard assay

Degradation experiments with isoflavones (10^{-4} M), isolation of products and incubation conditions were carried out according to earlier reports [2, 3].

Large scale incubations

Fungal mycelium (80 g) and 100 mg isoflavone were incubated in 2 l potassium phosphate buffer (pH 7.5, 0.05 M) until maximum production of product (monitored by TLC or HPLC). Product was isolated by ether extraction of the medium and purified by chromatographic techniques. Yield: 80-90%.

Demethylation of isoflavones

Isoflavones were O-demethylated with BBr₃ in dry methylenechloride according to [6]. Hydroxyisoflavones can be recovered quantitatively. All products were characterized as previously reported [3, 4].

Chromatography

TLC on silica gel was performed with the solvent S₁: dichloromethane/methanol = 15:1. Isolation of isoflavones by ladder column chromatography was by previous methods [4]. The HPLC separation [4] was carried out with the gradient of 20% B to 60% B in (A + B) in 35 min with A being 3% acetic acid and B acetonitril.

¹⁴C-Labelled isoflavones

¹⁴C-labelled samples of biochanin A and formononetin were from previous studies [4, 5]. Detection and measurement of ¹⁴C-substrates have been described [3]. All other isoflavones were from the institute's collection.

Acknowledgement

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Kinetin-Mediated Stimulation of Accumulation of Buckwheat Flavonoids in the Dark

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Anthocyanins, Flavonoids, Accumulation, Kinetics, [¹⁴C]-l-phenylalanine, *Fagopyrum esculentum*

A short treatment of excised buckwheat cotyledons with a solution of kinetin led to an up to 9-fold stimulation of anthocyanin biosynthesis, to an about 50 percent increase in the accumulation of rutin, and to an about 80 percent increase, on the average, in the accumulation of C-glycosylflavones in the treated material during its posttreatment incubation in the dark. When the treated cotyledons were incubated in a solution of L-phenylalanine anthocyanin accumulation in the dark practically attained the same high level as it was observed in the illuminated cotyledons fed with exogenous L-phenylalanine. In experiments with [¹⁴C]-labelled L-phenylalanine kinetin induced a sharp rise in the labelling (resp. in the utilization of exogenous substrate for biosynthesis) of anthocyanins and rutin in the dark and a slight increase in the radioactivity of C-glycosylflavones. Similar labelling changes occurred in the illuminated cotyledons. However, state as a result the relative portion of flavonoids formed from exogenous L-phenylalanine under these conditions showed a decrease as compared with the ratio of precursor use in the untreated cotyledons. The results show that low accumulation rates of anthocyanins and other flavonoids in the dark are conditioned by the limited access of substrate (L-phenylalanine) molecules normally controlled at the substrate rather than at the enzymic level.

Introduction

It is becoming increasingly evident that a change in the structure and properties of cell membranes is the first or an early step in the photocontrol of plant growth, development, and metabolism including biosynthesis of flavonoids [1, 2]. Resulting from treatments with kinetin, *n*-propyl, dimethylsulfoxide and other reagents which are believed to increase membrane permeability, considerable stimulation of anthocyanin formation was obtained in dark-grown material [3-10], exogenous shikimic and cinnamic acids promoted intense accumulation of anthocyanins in red cabbage seedlings in the dark while remaining without effect in the untreated material [4], and incorporation of exogenous L-phenylalanine into phenolic units of poplar xylem lignin in the dark became identical to that in the light [11]. By contrast, Ca²⁺ ions, which are known as membrane stabilizers, reduced or completely prevented the effects of kinetin and *n*-propylal as well as the similar effects of the light [6, 11].

These data suggest that light acts through the modification of membrane permeability, improving

conditions for intracellular transport and facilitating passage of substrate molecules through membrane barriers to the site of flavonoid biosynthesis. Further they allow to conclude that the main limitation to the formation of flavonoids in plant material unexposed to light is substrate availability rather than the activity of enzymes involved in that biosynthesis (see also [12-14]).

In this paper new supporting evidence for the limiting role of substrate availability in the synthesis of flavonoids in dark-grown material is presented. Using preliminary short treatments with kinetin and subsequent incubation of material in a solution of L-phenylalanine we were able to show that in buckwheat tissues with increased membrane permeability, both endogenous and exogenous substrates became much more accessible for flavonoid biosynthesis resulting in that the accumulation of these compounds in the dark could practically reach the level characteristic of their production under continuous illumination.

Materials and Methods

Plant material and treatment procedures

The experiments were carried out with isolated buckwheat (*Fagopyrum esculentum* Moench) cotyle-

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NEW ISOFLAVONES, INHIBITING CATECHOL-O-METHYLTRANSFERASE
PRODUCED BY *STREPTOMYCES*

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In the screening of catechol-O-methyltransferase inhibitors in streptomyces culture filtrates, three new isoflavones were isolated. Their structures were shown to be 3',5,7-trihydroxy-4',6-dimethoxyisoflavone (I), 3',5,7-trihydroxy-4',8-dimethoxyisoflavone (II), 3',8-dihydroxy-4',6,7-trimethoxyisoflavone (III). I and II inhibited both catechol-O-methyltransferase and dopa decarboxylase, and showed hypotensive action. III was a specific inhibitor of catechol-O-methyltransferase, and showed no hypotensive action.

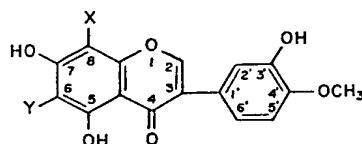
In 1958, AXELROD and TOMCHICK¹⁾ found an enzyme system which catalyses the transfer of the methyl group from S-adenosylmethionine to one of the phenolic hydroxyl groups of epinephrine and other catechols, and the enzyme has been purified from rat and human liver. This enzyme reaction is involved in extraneural inactivation of norepinephrine, and the inhibition of this route of catecholamine metabolism has been the subject of considerable research interest. Thus inhibitors found in various known compounds have been studied^{2,3)}. We were interested in the biological activity of specific inhibitors of this enzyme especially their effect on blood pressure and we screened the activity of culture filtrates of microorganisms inhibiting this enzyme. In previous papers^{7,8)}, we reported isolation of methylspinazarin, dihydromethylspinazarin from streptomyces and isolation from fungi of 7-O-methylspinochrome B and 6-(3-hydroxy-*n*-butyl)-7-O-methylspinochrome B which inhibit catechol-O-methyltransferase. In this paper, we report the isolation of isoflavone compounds inhibiting this enzyme from streptomyces. The action of one of them was specific to this enzyme and did not show hypotensive effect on spontaneously hypotensive rats.

The method described by NIKODEJEVIC *et al.*⁹⁾ was employed to determine the activity of streptomyces culture filtrates in inhibiting catechol-O-methyltransferase. Then, addition of 0.1 ml of a twice diluted culture filtrate of *Streptomyces roseolus* (ISP 5174) to the reaction mixture for the enzyme assay showed a significant inhibition. The activity was produced in media containing one of various carbon sources and plant nitrogen sources such as soybean meal, cotton seed meal or corn steep liquor. The following medium was selected as one of suitable media for the production: glucose 1.0 %, starch 2.0 %, defatted soybean meal 2.0 %, yeast extract 0.25 %, CaCO₃ 0.35 %, CuSO₄·5H₂O 0.0005 %, MnCl₂·4H₂O 0.0005 %, ZnSO₄·7H₂O 0.0005 %, pH 7.4 before sterilization. When cultured in this medium with or without yeast extract 0.5 % or corn steep liquor 0.5 %, addition of 0.05 ml of the twice diluted culture filtrate taken after 5 days on a reciprocating shaking machine at 27°C, showed from 50 to 65 % inhibition of the enzyme which catalysed the transfer of the ³H-methyl group from ³H-methyl-labeled S-adenosylmethionine.

methionine to one of catechol hydroxyl groups of epinephrine.

The activity in the culture filtrate was extracted with butyl acetate at pH 2.0, and that in the mycelial cake was extracted with methanol. The methanol was evaporated under reduced pressure, and the activity in the concentrate was extracted with butyl acetate at pH 2.0. Butyl acetate extracts thus obtained were combined and evaporated under reduced pressure, yielding a brownish oily material. After washing with petroleum ether, the residue was dissolved in acetone and the acetone solution was evaporated and dried to a brownish powder. It was dissolved in benzene-acetone (10 : 1) and subjected to silica gel column chromatography developed with the same solvent. Then, the presence of three active materials was clarified. The compound I which appeared in the earliest active peak was crystallized from acetone-*n*-hexane to give pale yellowish needles, m.p. 176°C. The compound II in the second active peak was crystallized from hot benzene to give pale yellowish needles, m.p. 197~198°C. The compound III which was most slowly eluted was crystallized from acetone-*n*-hexane to give colorless needles, m.p. 215°C.

These compounds were soluble in alkaline water, methanol, acetone, slightly soluble in butanol, ethyl acetate, butyl acetate. The *R_f* values on a thin-layer chromatography using silica gel were as follows: I 0.30, II 0.25 and III 0.19 with benzene-acetone (5 : 1); I 0.50, II 0.30, III 0.31 with chloroform-methanol (40 : 1); I 0.65, II 0.60, III 0.50 with benzene-ethyl acetate (1 : 1).



I: X = H, Y = OCH₃
II: X = OCH₃, Y = H

Compound I has molecular formula C₁₇H₁₄O₇ (MW 330.28). Found: C, 61.92; H, 4.31; O, 33.28. Calcd.: C, 61.82; H, 4.27; O, 33.91. *M*⁺, *m/e* 330. *ν*_{KBr}: 3500, 1655, 1630, 1580, 1520, 1480, 830 and 815 cm⁻¹. *λ*_{max}^{EIOH} (log *ε*): 269 nm (4.31) and 295 nm (shoulder).

Compound II has molecular formula C₁₇H₁₄O₇ (MW 330.28). Found: C, 61.63; H, 4.50; O, 34.31. Calcd.: C, 61.82; H, 4.27; O, 33.91. *M*⁺, *m/e* 330. *ν*_{KBr}: 3450, 1655, 1620, 1580, 1515, 825 and 810 cm⁻¹. *λ*_{max}^{EIOH} (log *ε*): 269 nm (4.31) and 295 nm (shoulder).

Compound III has molecular formula C₁₈H₁₆O₇ (MW 344.31). Found: C, 62.86; H, 4.79; O, 32.35. Calcd.: C, 62.79; H, 4.68; O, 32.53. *M*⁺, *m/e* 344. *ν*_{KBr}: 3500, 1650 (shoulder), 1615, 1580, 1515, 865 and 854 cm⁻¹. *λ*_{max}^{EIOH} (log *ε*): 268 nm (4.31) and 295 nm (shoulder).

The preliminary chemical studies suggested that they were structurally related compounds. By oxidative degradation with alkaline hydrogen peroxide they gave 3-hydroxy-4-methoxybenzoic acid which was identified by direct comparison of IR spectra with an authentic sample. The UV spectra suggested that they would have isoflavone structures rather than flavone, and the NMR spectra indicated a singlet proton signal at δ 8.3~8.4 in hexadeuterodimethylsulfoxide (DMSO-d₆), which was characteristic to C₂-proton in isoflavone compounds.

Compound I gave positive ferric chloride (dark blue) and GIBBS (violet) tests for the phenolic hydroxyl group. The NMR spectrum in DMSO-d₆ showed the presence of two methoxyl groups at δ 3.78 and 3.82, three hydroxyl protons at δ 13.04 (singlet, hydrogen bonded), *ca.* 10.8 and 9.1, the C₂-proton at δ 8.33, a singlet proton on A-ring at δ 6.52 and three aromatic

and positive GIBBS (blue) tests. The NMR spectrum in DMSO-d₂ showed the presence of three methoxyl groups at δ 3.90, 3.83 and 3.81, two hydroxyl protons at *ca.* δ 10.0 and 9.0, the C₂-proton at δ 8.39, a singlet proton on A-ring at δ 7.07 and three aromatic protons on B-ring

at δ 7.07 (1H) and 6.98 (2H). There was no hydrogen-bonded hydroxyl proton. Compound III gave the diacetyl derivative, m.p. 165°C. The NMR spectrum in CDCl_3 showed the aromatic proton of A-ring at δ 7.58 (singlet) which was isolated from three aromatic protons of B-ring at δ 7.40 (double doublets, $J=2.0$ and 8.5 Hz), 7.30 (doublet, $J=2.0$ Hz) and 7.02 (doublet, $J=8.5$ Hz). Nuclear OVERHAUSER effect was observed between the δ 7.58 proton and one of the methoxyl signals (3.98), +33 % $\text{CH}(\text{OCH}_3)$. The result suggested that one of the methoxyl groups is present at *ortho* position of the δ 7.58 proton. Addition of aluminium chloride did not cause UV shift as expected from negative ferric chloride reaction. On these bases, structure of compound III should be either 3', 8-hydroxy-4', 6, 7-trimethoxyisoflavone (III, $\text{R}=\text{CH}_3$, $\text{R}'=\text{H}$) or 3', 7-hydroxy-4', 6, 8-trimethoxyisoflavone (III', $\text{R}=\text{H}$, $\text{R}'=\text{CH}_3$). Addition of anhydrous sodium acetate to ethanolic solution of compound III caused a red shift of the main UV absorption band by 17 nm which was claimed to be characteristic of 7-hydroxyisoflavones.¹²⁾ Therefore, we initially preferred 3', 7-dihydroxy-4', 6, 8-trimethoxyisoflavone structure for the compound III. Alternative structure, 3', 8-dihydroxy-4', 6, 7-trimethoxyisoflavone, however, has not been excluded. The 3', 7-dihydroxy-4', 6, 8-trimethoxyisoflavone (III', $\text{R}=\text{H}$, $\text{R}'=\text{CH}_3$) has now been synthesized by H. SUGINOME *et al.*, the details of which will be reported in a forthcoming paper,¹³⁾ and it was found that the synthetic compound was not identical with the natural compound III. Therefore compound III should be correctly formulated as 3', 8-dihydroxy-4', 6, 7-trimethoxyisoflavone (III, $\text{R}=\text{CH}_3$, $\text{R}'=\text{H}$). This was confirmed by a direct comparison of dimethyl ether of the compound (III) and dimethyl ether of the synthetic 3', 7-dihydroxy-4', 6, 8-trimethoxyisoflavone, which proved that two dimethyl derivatives were identical.

Compounds I, II and III are new species of isoflavones which were isolated from streptomyces for the first time. A structure of the compound I was once reported by MORITA *et al.*,¹⁴⁾ but these authors' product was not identical with I. Hence, it should have a different structure.

Compounds I, II and III inhibited catechol-O-methyltransferase strongly *in vitro*. The ID_{50} was 0.70 $\mu\text{g/ml}$ (2.11×10^{-6} M), 2.0 $\mu\text{g/ml}$ (6.00×10^{-6} M) and 0.2 $\mu\text{g/ml}$ (5.81×10^{-7} M), respectively. Their methylated derivatives did not show any inhibition at 100 $\mu\text{g/ml}$, but their acetylated derivatives showed 50 % inhibition at 70 $\mu\text{g/ml}$ (1.55×10^{-4} M), 1.6 $\mu\text{g/ml}$ (3.55×10^{-6} M) and 1.0 $\mu\text{g/ml}$ (2.15×10^{-6} M) respectively.

The kinetics were studied using the enzyme which was partially purified by a modified method described by BALL and his associates.⁴⁾ The results are shown in Table 1. Compound I showed non-competitive type of inhibition, but compounds II and III showed mixed type of inhibition against epinephrine as substrate. Non-competitive type of inhibition was obtained against S-adenosylmethionine with these three compounds. Very little change of inhibition was observed under various concentrations of magnesium ions, from 3.1×10^{-4} M to 2.0×10^{-2} M.

The effects of compounds I, II and III on tyrosine hydroxylase, dopa decarboxylase and dopamine β -hydroxylase were also studied. In the reaction mixture described by LOVENBERG¹⁵⁾ preparing the enzyme solution as described by DAVIS and AWAPARA¹⁶⁾, dopa decarboxylase was inhibited by 5.0 $\mu\text{g/ml}$ (1.52×10^{-5} M) (ID_{50}) of compound II and by 12.5 $\mu\text{g/ml}$ (3.79×10^{-5} M) of compound I but not by compound III (100 $\mu\text{g/ml}$). The compounds I and II inhibited histidine decarboxylase prepared from rat embryo in the reaction mixture as described previously:¹⁷⁾

hydroxyl proton. Compound spectrum in CDCl_3 showed the 1 from three aromatic protons (doublet, $J=2.0$ Hz) and 7.02 between the δ 7.58 proton and sult suggested that one of the on. Addition of aluminium ic chloride reaction. On these xy-4', 6, 7-trimethoxyisoflavone avone (III', $R=H$, $R'=CH_3$). compound III caused a red to be characteristic of 7-hydroxy-xy-4', 6, 8-trimethoxyisoflavone xy-4', 6, 7-trimethoxyisoflavone, methoxyisoflavone (III', $R=H$, details of which will be reported compound was not identical ould be correctly formulated as l). This was confirmed by a dimethyl ether of the synthetic two dimethyl derivatives were

ich were isolated from strepto- nce reported by MORITA *et al.*¹⁴, should have a different struc- ase strongly *in vitro*. The ID_{50} ml (5.81×10^{-7} M), respectively. 100 $\mu\text{g}/\text{ml}$, but their acetylated .6 $\mu\text{g}/\text{ml}$ (3.55×10^{-6} M) and 1.0

partially purified by a modified shown in Table 1. Compound I and III showed mixed type of type of inhibition was obtained little change of inhibition was 3.1×10^{-4} M to 2.0×10^{-2} M. ylase, dopa decarboxylase and xture described by LOVENBERG¹⁵. PARA¹⁶, dopa decarboxylase was l by 12.5 $\mu\text{g}/\text{ml}$ (3.79×10^{-5} M) of ands I and II inhibited histidine ure as described previously¹⁷:

50 % inhibition concentration of I was 1.8×10^{-3} M and that of II was 4.5×10^{-6} M. They did not inhibit the other enzymes at 100 $\mu\text{g}/\text{ml}$. The intravenous administration of 200 mg/kg of each substance to mice did not show any toxic sign. Antimicrobial activity of these compounds was not observed at 100 $\mu\text{g}/\text{ml}$.

Table 1. Inhibition of catechol-O-methyltransferase by compounds I, II and III

	Substrate			
	Epinephrine ($K_m=8.3 \times 10^{-4}$ M)		S-Adenosylmethionine ($K_m=1.3 \times 10^{-4}$ M)	
	Type of inhibition	K_i	Type of inhibition	K_i
I	non-competitive	1.7×10^{-6} M	non-competitive	1.7×10^{-6} M
II	mixed	3.3×10^{-6} M	non-competitive	9.1×10^{-6} M
III	mixed	1.5×10^{-7} M	non-competitive	3.9×10^{-7} M

Measurement of catechol-O-methyltransferase activity was carried out by the modified method of NIKODEJEVIC *et al.*¹⁹ The concentrations of epinephrine were varied from 1.6×10^{-4} M to 2.5×10^{-3} M under the concentration of 7.5×10^{-4} M of S-adenosylmethionine. Similarly, the concentrations of S-adenosylmethionine were varied from 4.7×10^{-5} M to 7.5×10^{-4} M under the concentration of 2.5×10^{-3} M of epinephrine.

Fig. 1. Effect of intraperitoneal injection of compound I on blood pressure of SH-rats. Mean depression percentage (4 rats) of each dose. Bars show the standard error of 4 rats.

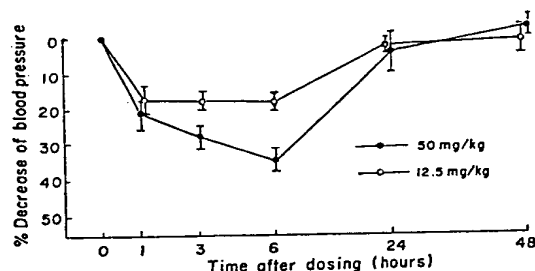
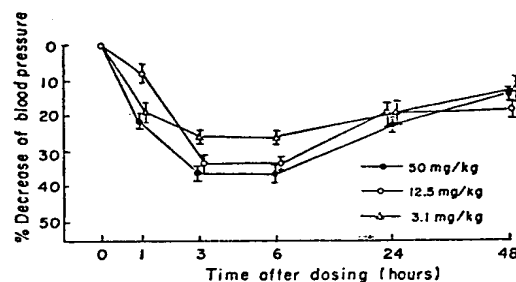


Fig. 2. Effect of intraperitoneal injection of compound II on blood pressure of SH-rats. Mean depression percentage (4 rats) of each dose. Bars show the standard error of 4 rats.



Hypotensive effect was shown by the compounds I and II, and the latter showed a strong effect. When 50 mg/kg of I was intraperitoneally injected to spontaneously hypotensive rats (4 rats were injected in each dose), the percent blood pressure decrease after various hours of the injection compared with the pressure before the injection is shown in Figs. 1 and 2. The marked decrease in blood pressure was observed 3~6 hours after the injection of both compounds. The compound II showed the stronger effect than the compound I and the significant decrease was shown even by 3.1 mg/kg during 3~24 hours after the injection. The intraperitoneal injection of 50 mg/kg of III did not show a significant decrease of blood pressure. As described above, among compounds I, II, and III, the activity inhibiting catechol-O-methyltransferase was the strongest in III and II showed the strongest activity in inhibiting dopa decarboxylase. It suggests that inhibition of catechol-O-methyltransferase would not be related to the hypotensive action, but inhibition of dopa decarboxylase might be the reason for the hypotensive effect.

As described in this paper, we found three new isoflavones in streptomyces culture filtrate. Such compounds could not be found in media before inoculation of the strain ISP 5174. However, it is not certain whether streptomyces can synthesize the isoflavone skeleton *de novo*. It is possible that isoflavone compounds in medium ingredients such as soybean meal might be converted to the compounds I, II and III by streptomyces.

Experimental

Equipments for physical determination:

UV was measured by Hitachi UV spectrometer EPS-3T, IR by Hitachi Infrared spectrometer EPI-S2, NMR by Varian HA-100D, mass analysis by Hitachi RMU-6M.

Method of assay for activity inhibiting catechol-O-methyltransferase:

The reaction mixture consisted of distilled water 0.125 ml, 0.1 M phosphate buffer at pH 8.0 0.05 ml, 0.1 M $MgCl_2$ 0.1 ml, 0.05 M epinephrine 0.05 ml, 0.05 mM 3H -methyl-labeled S-adenosylmethionine (2.2×10^5 cpm) 0.075 ml, a test sample or distilled water 0.05 ml, the enzyme solution 0.05 ml. The reaction mixture except enzyme was mixed at 0°C and the reaction was started after addition of the enzyme solution and the incubation at 37°C was continued for 20 minutes. The reaction was stopped by addition of 1.0 ml of 0.5 M borate buffer at pH 10.0 and the reacted mixture was extracted with toluene-isoamylalcohol (3:2) and the radioactivity in the solvent layer was counted by Beckmann scintillation counter LS-250.

The enzyme solution was prepared from rat liver. It was homogenized with 3 volume of 0.25 M sucrose and centrifuged at 105,000 g. This supernatant was divided into each 2 ml and kept in frozen. Before use, it was diluted 10 times with distilled water and employed. Epinephrine solution was prepared as follows: 832.5 mg of epinephrine bitartrate (purchased from Tokyo Kasei Co.) was dissolved in 50 ml of 0.1 N HCl and pH was adjusted to 5.0 and kept in frozen. The percent inhibition was calculated from the radioactivity as follows $\left(1 - \frac{A-C}{B-C}\right) \times 100$, where B is that without inhibitor, A is that with inhibitor and C is that without the enzyme. The culture filtrate was heated at 100°C for 3 minutes before assay.

For kinetic study, catechol-O-methyltransferase was purified as follows: Rat liver (31.25 g) was homogenized with 2 volumes of 0.25 M sucrose and centrifuged at 105,000 g for 1 hour. The supernatant was made pH 5.0 by addition of acetic acid and the precipitate was removed by 10 minutes centrifugation at 10,000 g. To this supernatant 0.5 M phosphate buffer at pH 7.8 was added until pH became 6.8 (the total volume became 61 ml). To this solution 10.7 g of ammonium sulfate was added and the precipitate was removed by centrifugation at 10,000 g for 10 minutes. To the supernatant 15.3 g of ammonium sulfate (to 65 % saturation) was added and centrifuged at 10,000 g for 20 minutes. The precipitate was dissolved in 2 mM phosphate buffer (11.5 ml) at pH 7.4 and subjected to Sephadex G 100 column (3×70 cm) chromatography developed with the same buffer. The effluent was cut into each 5g, and the active effluent (from 20th to 28th fractions) was passed through CM-Sephadex column (2×6 cm) equilibrated with 2 mM phosphate buffer at pH 6.7. The effluent was collected in 4-ml fractions. The active fractions (3rd~10th fractions) were collected. About 20 fold purification was accomplished by this method. This partially purified enzyme solution was employed for the kinetic study.

Extraction and isolation of compounds I, II, and III:

S. roseolus ISP 5174 was shake-cultured in a medium containing glucose 1.0 %, starch 2.0 %, defatted soybean meal 2.0 %, NaCl 0.25 %, $CaCO_3$ 0.35 %, $CuSO_4 \cdot 5H_2O$ 0.0005 %, $MnCl_2 \cdot 4H_2O$ 0.0005 %, $ZnSO_4 \cdot 7H_2O$ 0.005 % (pH 7.4 before sterilization). After 3 days shaking culture at 27°C, 500 ml of the culture broth was inoculated to 12 liters of the same medium placed in a jar fermentor and 1.2 ml of silicone resin was added to suppress the foaming. The fermentation was continued for 105 hours at 27°C under aeration of 12 liters/minute and 250 rpm stirring. The culture broth of 4 fermentors was combined and centrifuged at 2,500 rpm. The

liquid part (40 liters) and the wet solid part (5 kg) were thus separated. The wet solid was extracted with 5 liters of methanol. Addition of 0.05 ml of the 4 times diluted methanol solution (4.8 liters) or of the twice diluted culture filtrate to the reaction mixture for assay of an inhibitor of catechol-O-methyltransferase showed 50% inhibition. This methanol solution was concentrated to 500 ml under reduced pressure and added to the liquid part of the culture broth and extracted with 40 liters of butyl acetate at pH 2.0. The evaporation of the butyl acetate solution under reduced pressure gave 80 g of oily material. It was washed with petroleum ether yielding 35.0 g of brown powder ($ID_{50}=111 \mu\text{g}$). The powder was dissolved in 300 ml of acetone and the insoluble part was removed. To the acetone solution, 90 g of silica gel (Mallinckrodt, Silicic Acid AR 100 mesh) was added and dried under reduced pressure. This powder was placed on the top of the silica gel column (6.5 \times 58.0 cm), and the column was developed with the same solvent. A yellow powder (790 mg) containing compound I was obtained by evaporation of the first active fraction (800 ml) under reduced pressure. Another active yellow powder (250 mg) containing compound II was obtained from the second active eluate (1,500 ml) and the active brown powder (150 mg) containing compound III was obtained from the third active fraction (1,500 ml). These powders showed 50% inhibition of catechol-O-methyltransferase in the following doses: I powder 20 μg ; II powder 50 μg ; III powder 2.0 μg .

The I powder was dissolved in 10 ml of methanol and passed through Sephadex LH-20 (500 ml). The activity appeared in one peak (25 ml) and the eluate was evaporated to dryness under reduced pressure. The powder was dissolved with 5 ml of acetone and 20 ml of *n*-hexane was added. After overnight, I crystallized: 18.5 mg of pale yellow needle crystals.

The II powder described above was subjected to Sephadex LH-20 chromatography similar to that described above. The powder thus obtained was dissolved in 4 ml of benzene at 60°C. Then, after 1 week at room temperature, 20 mg of II crystals was obtained.

The III powder described above was mixed with 3 times weight of alumina and placed in a column and the compound III was dissolved in methanol which was passed through the column. The brown-colored impurity was removed by this method. The methanol solution was passed through Sephadex LH-20 column (500 ml) and the active eluate was evaporated to dryness. The crystallization by the same method as used for compound I gave 11.5 mg of colorless needle crystals of III.

Method of measuring the activity in inhibiting dopa decarboxylase^{15,16}: The reaction mixture consisted of L-dopa 1×10^{-3} M, pyridoxal phosphate 7.5×10^{-3} M, 0.05 ml of dopa decarboxylase solution, phosphate buffer (pH 6.9) 0.03 M, iproniazid 1×10^{-3} M, 0.1 ml of a test material, and the total volume was made 1.5 ml with distilled water. The reaction was started by addition of the enzyme solution. The incubation time at 37°C was 25 minutes. The reaction was stopped by heating at 100°C for 3 minutes. The reacted solution was passed through Amberlite CG-50 column (0.5 \times 7.5 cm) and dopamine adsorbed was eluted with 1.5 ml of 1 N acetic acid. The optical density at 279 nm of the eluate was read.

Dopa decarboxylase was prepared from rat liver. It was homogenized with 2 volumes of 0.003 M mercaptoethanol. The homogenate was centrifuged at 13,000 rpm for 20 minutes, and the precipitate was obtained by 0.45~0.55 saturation of ammonium sulfate. It was dissolved in 0.003 M mercaptoethanol. The concentration of the enzyme solution was adjusted as its addition of 0.05 ml gave optical density 0.3 of dopamine. Generally, the protein content of this enzyme solution was 1.0 mg/ml.

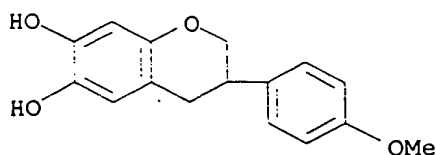
Testing the hypotensive effect: Four spontaneously hypertensive rats (male) of 15~20 weeks age were employed for each dosis. One, 3, 6, 24 and 48 hours of intraperitoneal injection of 50 mg/kg or 12.5 mg/kg of compound I, 50 mg/kg, 12.5 mg/kg or 3.1 mg/kg of compound II and 50 mg/kg of compound III, the blood pressure was measured by the plethysmographic tail method. Percent decrease from the pressure before injection was calculated (Figs. 1 and 2).

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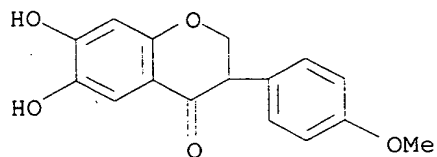
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LS ANSWER 1 OF 19 CA COPYRIGHT 2000 ACS
AN 128:164027 CA
TI Isoflavonoids as inhibitors of lipid peroxidation and quenchers of singlet oxygen
AU Briviba, Karlis; Sies, Helmut; Sepulveda-Boza, Silvia; Zilliken, Friedrich W.
CS Heinrich Heine University, Dusseldorf, Germany
SO Antioxid. Health Dis. (1998), 7(Flavonoids in Health and Disease), 295-302
CODEN: AHDIEQ
PB Marcel Dekker, Inc.
DT Journal
LA English
AB This report examines the inhibition of microsomal lipid peroxidn. and the ability of singlet oxygen quenching of some new isoflavones and isoflavans and compares the antioxidant properties of these isoflavonoids with established antioxidants.
IT 76397-87-0
RL: BAC (Biological activity or effector, except adverse); BIOL (Biological study)
(isoflavonoids as inhibitors of lipid peroxidn. and quenchers of singlet oxygen)
RN 76397-87-0 CA
CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA INDEX NAME)

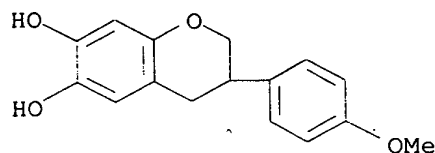


01

L5 ANSWER 2 OF 19 CA COPYRIGHT 2000 ACS
 AN 122:156104 CA
 TI Structure-activity relationships among isoflavonoids with regard to their antifungal properties
 AU Weidenboerner, Martin; Jha, Hem Chandra
 CS Institut fur Lebensmitteltechnologie, Universitat Bonn, Bonn, 53117, Germany
 SO Mycol. Res. (1994), 98(12), 1376-8
 CODEN: MYCRER; ISSN: 0953-7562
 DT Journal
 LA English
 AB In order to establish a structure-activity relationship in the class of isoflavonoids, 16 differently substituted isoflavonoids were tested against *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium oxysporum* and *Trichoderma harzianum*. The isoflavanones, 6,7-dihydroxy-4'-methoxy- and 7-hydroxy-8,4'-dimethylisoflavanone, showed highest antifungal activity in the case of *C. herbarum* as test fungus. The unreduced structure of the isoflavones has less inhibitory effect on the growth of the test fungi, whereas the completely reduced isoflavones, i.e., the isoflavans, showed only a very weak activity.
 IT 76397-85-8 76397-87-0
 RL: BAC (Biological activity or effector, except adverse); BIOL (Biological study)
 (structure-activity relationships among isoflavonoids with regard to their antifungal properties)
 RN 76397-85-8 CA
 CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)- (9CI) (CA INDEX NAME)



RN 76397-87-0 CA
 CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA INDEX NAME)

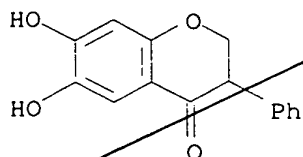


L5 ANSWER 3 OF 19 CA COPYRIGHT 2000 ACS
 AN 118:124232 CA
 TI Synthesis and antioxidant activity of isoflavones and isoflavanones
 AU Bulut, Mustafa
 CS Fac. Nat. Sci. Lit., Univ. Marmara, Kadikoy, Turk.
 SO Chim. Acta Turc. (1992), 19(2), 121-8
 CODEN: CATUA9; ISSN: 0379-5896
 DT Journal
 LA German
 AB Fifteen hydroxyisoflavones were prepd. and reduced to the hydroxyisoflavanones. Polyhydroxylated isoflavones, particularly 5,7,3',4'-tetrahydroxy- and 6,7-dihydroxyisoflavone, had antioxidant activity, but natural 5,7-dihydroxyisoflavones are not particularly good antioxidants. The isoflavanones were more active than the isoflavones, particularly the 6-hydroxy derivs.
 IT 77229-73-3P

RL: SPN (Synthetic preparation); PREP (Preparation)
(prepn. and antioxidant activity of)

RN 77229-73-3 CA

CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-phenyl- (9CI) (CA
INDEX NAME)



L5 ANSWER 4 OF 19 CA COPYRIGHT 2000 ACS

AN 118:101686 CA

TI Novel synthesis of isoflavones and study of the pharmacological properties
of their derivatives

AU Bulut, Mustafa

CS Natur. Literat. Sci. Fac., Univ. Marmara, Istanbul, Turk.

SO Chim. Acta Turc. (1992), Volume Date 1991, 19(1), 17-26

CODEN: CATUA9; ISSN: 0379-5896

DT Journal

LA German

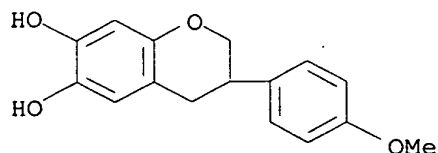
AB Dihydroxydeoxybenzoins, chromones, 3-phenylcoumarins, 3-phenylchromones,
and isoflavans were tested for antioxidant activity in vitamin E-free
lard. o-Dihydroxy substitution in the arom. ring of the benzopyran moiety
led to high antioxidant activity. Some of the compds. were prepd. Thus,
3-phenylchromones were obtained by NaBH4-H3BO3 redn. of isoflavones and by
Dibal redn. of 3-phenylcoumarins.

IT 76397-87-0P 94105-89-2P 97148-44-2P

RL: SPN (Synthetic preparation); PREP (Preparation)
(prepn. and antioxidant activity of)

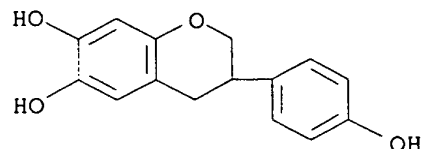
RN 76397-87-0 CA

CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA
INDEX NAME)



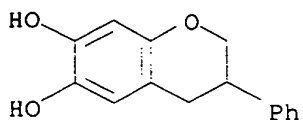
RN 94105-89-2 CA

CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-hydroxyphenyl)- (9CI) (CA
INDEX NAME)

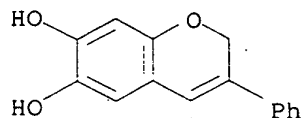


RN 97148-44-2 CA

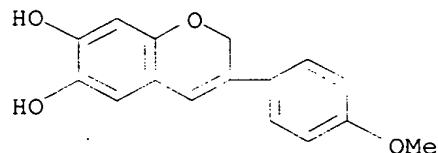
CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-phenyl- (9CI) (CA INDEX NAME)



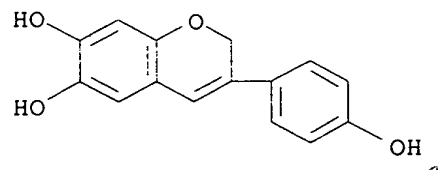
IT 145917-91-5P 145917-92-6P 145917-93-7P
 RL: RCT (Reactant); SPN (Synthetic preparation); PREP (Preparation)
 (prepn., redn., and antioxidant activity of)
 RN 145917-91-5 CA
 CN 2H-1-Benzopyran-6,7-diol, 3-phenyl- (9CI) (CA INDEX NAME)



RN 145917-92-6 CA
 CN 2H-1-Benzopyran-6,7-diol, 3-(4-methoxyphenyl)- (9CI) (CA INDEX NAME)

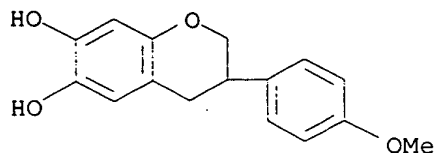


RN 145917-93-7 CA
 CN 2H-1-Benzopyran-6,7-diol, 3-(4-hydroxyphenyl)- (9CI) (CA INDEX NAME)

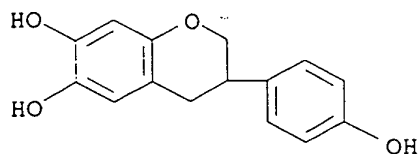


L5 ANSWER 5 OF 19 CA COPYRIGHT 2000 ACS
 AN 117:124019 CA
 TI New isoflavonoids as inhibitors of porcine 5-lipoxygenase
 AU Voss, Cornelia; Sepulveda-Boza, Silvia; Zilliken, Friedrich W.
 CS Inst. Physiol. Chem., Univ. Bonn, Bonn, 5300/1, Germany
 SO Biochem. Pharmacol. (1992), 44(1), 157-62
 CODEN: BCPCA6; ISSN: 0006-2952
 DT Journal
 LA English
 AB The inhibitory activity of new isoflavonoids on 5-lipoxygenase of porcine leukocytes was investigated. Isoflavans proved to be stronger inhibitors than isoflavones. The isoflavans contg. ortho-hydroxy groups in ring A showed the lowest Ki values (0.8-50 .mu.M). In comparison, isoflavans with meta-dihydroxy groups exhibited Ki values higher than 150 .mu.M. The effect of com. antioxidants was tested also on porcine 5-lipoxygenase. Butylated hydroxyanisole (Ki: 25 .mu.M) and butylated hydroxytoluene (Ki: 55 .mu.M) revealed moderate inhibitory activity, whereas L-ascorbic acid, L-ascorbyl palmitate, DL-.alpha.-tocopherol and n-Pr gallate showed weak inhibitory activities (Ki: 100-260 .mu.M).
 IT 76397-87-0 94105-89-2 116718-58-2
 RL: BIOL (Biological study)
 (5-lipoxygenase inhibition by, structure in relation to)

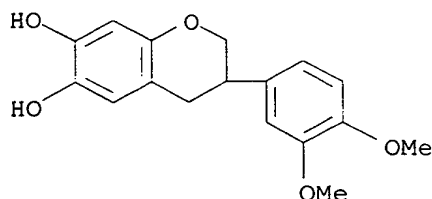
RN 76397-87-0 CA
CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA
INDEX NAME)



RN 94105-89-2 CA
CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-hydroxyphenyl)- (9CI) (CA
INDEX NAME)



RN 116718-58-2 CA
CN 2H-1-Benzopyran-6,7-diol, 3-(3,4-dimethoxyphenyl)-3,4-dihydro- (9CI) (CA
INDEX NAME)



L5 ANSWER 6 OF 19 CA COPYRIGHT 2000 ACS

AN 114:41246 CA

TI Control of storage fungi of the genus *Aspergillus* on legumes with
flavonoids and isoflavonoids

AU Weidenborner, M.; Hindorf, H.; Jha, H. C.

CS Inst. Pflanzenkrankheiten, Univ. Bonn, Bonn, D-5300, Fed. Rep. Ger.

SO Angew. Bot. (1990), 64(1-2), 175-90

CODEN: ANBTJ; ISSN: 0066-1759

DT Journal

LA German

AB Flavonoids, isoflavonoids, and their derivs. were screened for their
fungicidal effects against storage fungi of the genus *Aspergillus* on seeds
of soybean (*Glycine max*) and bean (*Phaseolus vulgaris*). A high redn. in
fungal infestation in bean seeds was achieved with the mixts.
flavanon/6,7-Dihydroxy-4'-methoxyisoflavan, 2% active ingredient (a.i.),
and 6,7-dihydroxy-4'-methoxyisoflavan/6,7-dihydroxy-3'-methylisoflavan, 2%
a.i., after 3 wk, with 87.9 and 87.1% redns., resp. A significant redn.
(69.9%) of infestation in soybeans was achieved with the combination of
the isoflavans 6,7-dihydroxy-4'-methoxyisoflavan/6,7-dihydroxy-3'-
methylisoflavan, 2% a.i., after 2 wk. Benomyl, at 1% a.i., caused similar
inhibition rates in both seeds.

IT 76397-85-8, 6,7-Dihydroxy-4'-methoxyisoflavanone

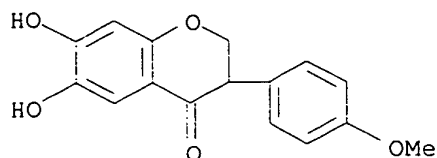
76397-87-0 131426-41-0

RL: BIOL (Biological study)

(*Aspergillus* spp. control with, on beans and soybeans)

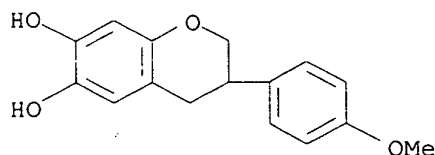
RN 76397-85-8 CA

CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)-
(9CI) (CA INDEX NAME)



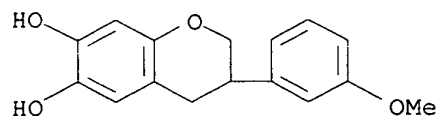
RN 76397-87-0 CA

CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA
INDEX NAME)



RN 131426-41-0 CA

CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(3-methoxyphenyl)- (9CI) (CA
INDEX NAME)



L5 ANSWER 7 OF 19 CA COPYRIGHT 2000 ACS

AN 113:94654 CA

TI Antifungal activity of isoflavonoids in different reduced stages on
Rhizoctonia solani and Sclerotium rolfsii

AU Weidenboerner, Martin; Hindorf, Holger; Jha, Hem Chandra; Tsotsonos,
Prodromos; Egge, Heinz

CS Inst. Pflanzenkrankheiten, Univ. Bonn, Bonn, D-5300, Fed. Rep. Ger.

SO Phytochemistry (1990), 29(3), 801-3

CODEN: PYTCAS; ISSN: 0031-9422

DT Journal

LA English

AB Two naturally occurring isoflavones, genistein and iochanin A, and their dihydroderivs. (isoflavanones), as well as 9 perhydrogenated isoflavones (isoflavans), were tested for their effects on mycelial growth of the 2 soil borne fungi Rhizoctonia solani and Sclerotium rolfsii. All the isoflavonoids of the biochanin A series showed high antifungal activity. Genistein isoflavan and the other isoflavans with 2 hydroxyl groups and one methoxy group were fungitoxic, while isoflavan with 2 or 3 methoxy groups were almost inactive.

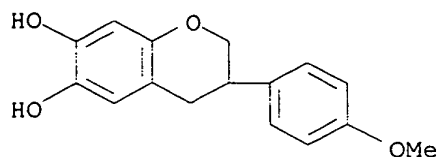
IT 76397-87-0

RL: BAC (Biological activity or effector, except adverse); BIOL
(Biological study)

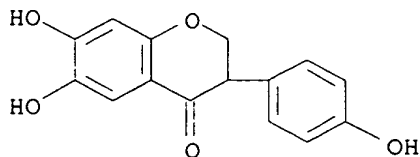
(antifungal activity of, structure in relation to)

RN 76397-87-0 CA

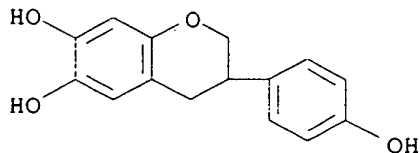
CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA
INDEX NAME)



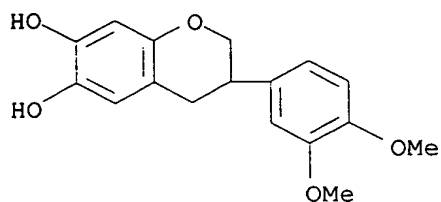
L5 ANSWER 8 OF 19 CA COPYRIGHT 2000 ACS
 AN 112:95337 CA
 TI Antifungal activity of isoflavonoids against storage fungi of the genus *Aspergillus*
 AU Weidenboerner, Martin; Hindorf, Holger; Jha, Hem Chandra; Tsotsonos, Prodromos; Egge, Heinz
 CS Inst. Pflanzenkrankh., Univ. Bonn, Bonn, D-5300/1, Fed. Rep. Ger.
 SO Phytochemistry (1989), 28(12), 3317-19
 CODEN: PYTCAS; ISSN: 0031-9422
 DT Journal
 LA English
 AB The fungicidal activity of 2 isoflavones, 1 isoflavanone and 7 isoflavans was tested in malt ext. broth against 5 storage fungi of the genus *Aspergillus*. While the isoflavones and the isoflavanone show only low activity, the 2 isoflavans 7,8-dihydroxy-4'-methoxyisoflavan and 6,7-dihydroxy-3'-methylisoflavan were highly inhibitory to *Aspergillus*. Structure-activity relationships are discussed.
 IT 94105-87-0 94105-89-2, 6,7,4'-Trihydroxyisoflavan
 116718-58-2
 RL: BAC (Biological activity or effector, except adverse); BIOL (Biological study)
 (antifungal activity of, against *Aspergillus*)
 RN 94105-87-0 CA
 CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-hydroxyphenyl)- (9CI) (CA INDEX NAME)



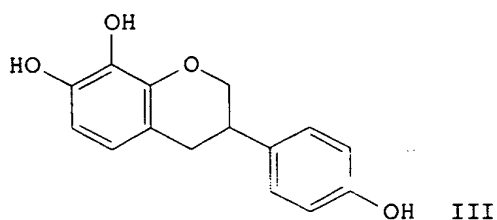
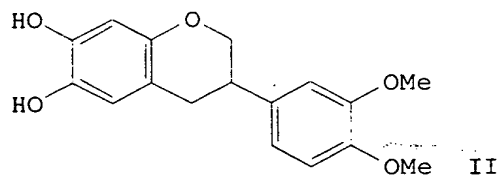
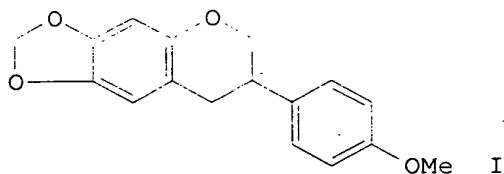
RN 94105-89-2 CA
 CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-hydroxyphenyl)- (9CI) (CA INDEX NAME)



RN 116718-58-2 CA
 CN 2H-1-Benzopyran-6,7-diol, 3-(3,4-dimethoxyphenyl)-3,4-dihydro- (9CI) (CA INDEX NAME)



L5 ANSWER 9 OF 19 CA COPYRIGHT 2000 ACS
 AN 112:69573 CA
 TI In-vitro versus in-vivo activities of new 5-lipoxygenase inhibitors with anti-inflammatory activity
 AU Montandon, J. B.; Zijlstra, F. J.; Wilson, J. H. P.; Grandjean, E. M.; Cicurel, L.
 CS Zyma SA, Nyon, 1260, Switz.
 SO Int. J. Tissue React. (1989), 11(3), 107-12
 CODEN: IJTEDP; ISSN: 0250-0868
 DT Journal
 LA English
 GI



AB The possible relationship between in-vitro inhibition of lipoxygenase (LO)/cyclooxygenase (CO) and in-vivo anti-inflammatory effects of compds. such as isoflavanes, Zy 16369 (I), Zy 16372 (II), and Zy 16681 (III), was investigated. The latter were all shown to be potent 5-LO inhibitors when tested in vitro on human peritoneal macrophages ($IC_{50} = 1-7 \mu M$). II and III also inhibited the 12- and 15-LO and, to a minor extent, the CO. In order to evaluate the anti-inflammatory and antiproliferative effects of these compds. in vivo they were applied topically to mice. No definite correlation could be made between the inhibition of the ear edema induced by arachidonic acid (AA), the inhibition of the epidermal ornithine-decarboxylase (ODC) activity induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), and the in-vitro activities of the compds. II appeared to inhibit the edema dose-dependently ($ED_{50} = 5 \mu M/ear$) and seemed to be the most potent among the 3 compds. tested and

slightly more potent than the ref. compd. nordihydroguaiaretic acid. As inhibitors of TPA-induced ODC, all 3 compds. exhibited comparable activity. These results suggest that the in-vitro effects of the compds. might be mediated by components other than AA metabolites, and/or be related to their specific kinetic patterns.

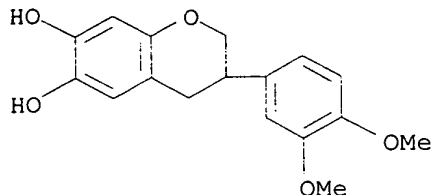
IT 116718-58-2, Zy 16372

RL: BIOL (Biological study)

(anti-inflammatory activity of and lipoxigenase/cyclooxygenase inhibition by)

RN 116718-58-2 CA

CN 2H-1-Benzopyran-6,7-diol, 3-(3,4-dimethoxyphenyl)-3,4-dihydro- (9CI) (CA INDEX NAME)



L5 ANSWER 10 OF 19 CA COPYRIGHT 2000 ACS

AN 109:209979 CA

TI Effects of alpha-tocopherol, its carboxylic acid chromane compound and two novel antioxidant isoflavanones on prostaglandin H synthase activity and autodeactivation

AU Seeger, Werner; Moser, Ulrike; Roka, Ladislaus

CS Dep. Intern. Med., Justus-Liebig-Univ., Giessen, D-6300, Fed. Rep. Ger.

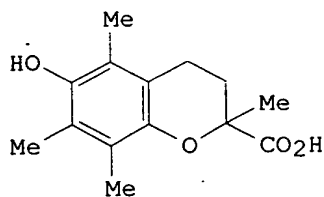
SO Naunyn-Schmiedeberg's Arch. Pharmacol. (1988), 338(1), 74-81

CODEN: NSAPCC; ISSN: 0028-1298

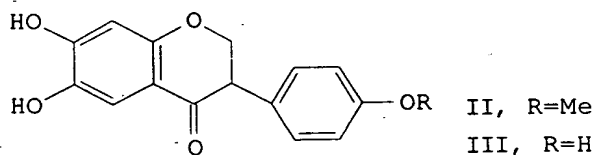
DT Journal

LA English

GI



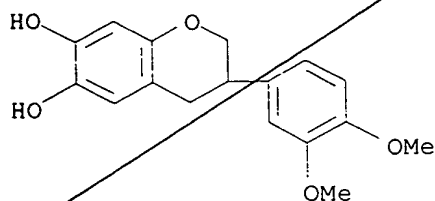
I



III, R=H

AB The effects of different antioxidants on ram vesicular gland microsomal prostaglandin H synthase activity were investigated in vitro. They were d,l-alpha-tocopherol, its carboxylic acid chromane compd. (I), phytol, alpha-tocopherol acetate, and 2 novel antioxidative isoflavanones obtained by methylation and/or hydrogenation of naturally occurring isoflavones from fermented soybeans, 6,7-dihydroxy-4'-methoxyisoflavanone (II) and 6,7,4'-trihydroxyisoflavanone (III). alpha-Tocopherol, its acetate, and phytol had no significant influence on the enzyme activity when applied in concns. 1 to 1000 microM. Trolox (100-1000 microM) and the 2 isoflavanones (5-50 and 10-100 microM) dose-dependently augmented the initial rate of O2 consumption and the total O2 uptake during prostaglandin H synthase incubation with arachidonic acid. These compds. also increased the formation of prostaglandin E2 and F2.alpha. from 14C-labeled AA and they markedly protected prostaglandin H synthase from rapid autodeactivation. These compds. may serve as cosubstrates to which the oxidizing equiv. are transferred which arise during the hydroperoxidase reaction of the enzyme.

14



L5 ANSWER 12 OF 19 CA COPYRIGHT 2000 ACS

AN 109:21889 CA

TI Fungicidal action of isoflavonoids on *Aspergillus* molds

AU Weidenborner, M.; Jha, H. C.; Hindorf, H.; Weltzien, H. C.; Zilliken, F.

CS Inst. Pflanzenkrankh., Univ. Bonn, Bonn, 5300/1, Fed. Rep. Ger.

SO Meded. Fac. Landbouwwet., Rijksuniv. Gent (1987), 52(3A), 933-42

CODEN: MFLRA3; ISSN: 0368-9697

DT Journal

LA German

AB The effect of a few selected isoflavonoids was tested in malt ext. soln. at 0.5, 2, and 8 .times. 10⁻⁴ mol/L on the mycelial growth of 5 fungi, *A. repens*, *A. amstelodami*, and *A. chevalieri* of the *A. glaucus* group, *A. flavus* of the *A. flavus* group and *A. petrakii* of the *A. ochraceus* group. To establish structure-activity relationship, besides unsubstituted isoflavonoids, methoxy- and hydroxyisoflavonoids were tested. Isoflavanone inhibited mycelial growth up to 57.1% in *A. amstelodami* at 2 .times. 10⁻⁴ mol/L. In the texasin-isoflavonoid (6,7-dihydroxy-4'-methoxyisoflavonone) series the isoflavan was the most effective one with growth inhibitions of 96.9% in *A. repens*, 97.9% in *A. amstelodami*, and 100% in *A. chevalieri* at 8 .times. 10⁻⁴ mol/L. Also, the coumestans as a further subgroup of natural isoflavonoids were tested. The influence of these substances was quite variable. While the 6,7,3',4'-tetrahydroxycoumestan always stimulated growth (*A. flavus* 22.5%), 3',4'-dihydroxycoumestan caused no significant differences in comparison with the control. 3',4'-Dihydroxy-5'-methoxycoumestan inhibited the growth of *A. repens* to 20.1% and the growth of *A. petrakii* to 9.2% at 0.5 .times. 10⁻⁴ mol/L.

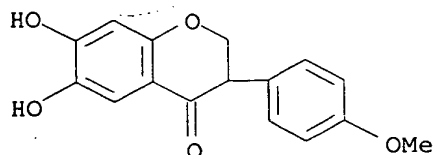
IT 76397-85-8 76397-87-0

RL: BIOL (Biological study)

(*Aspergillus* inhibition by)

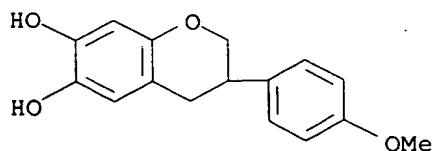
RN 76397-85-8 CA

CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)- (9CI) (CA INDEX NAME)

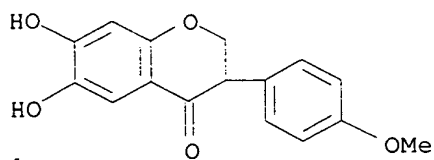


RN 76397-87-0 CA

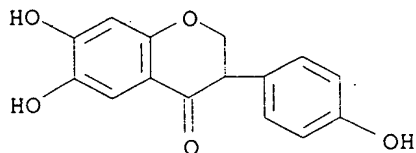
CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-methoxyphenyl)- (9CI) (CA INDEX NAME)



IT 76397-85-8, 6,7-Dihydroxy-4'-methoxyisoflavanone
 94105-87-0, 6,7,4'-Trihydroxyisoflavanone
 RL: BIOL (Biological study)
 (prostaglandin H synthase response to)
 RN 76397-85-8 CA
 CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-methoxyphenyl)-
 (9CI) (CA INDEX NAME)



RN 94105-87-0 CA
 CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-hydroxyphenyl)-
 (9CI) (CA INDEX NAME)



L5 ANSWER 11 OF 19 CA COPYRIGHT 2000 ACS

AN 109:149354 CA

TI Preparation and formulation of 3-aryl-3,4-dihydro-2H-1-benzopyrans useful
 in treatment of vascular diseases

IN Albert, Alban Imre; Zilliken, Friedrich W.

PA Zyma S. A., Switz.

SO Eur. Pat. Appl., 23 pp.

CODEN: EPXXDW

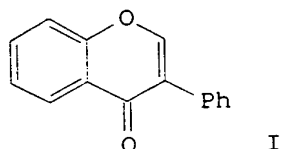
DT Patent

LA English

FAN.CNT 1

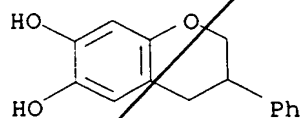
	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	EP 267155	A2	19880511	EP 1987-810620	19871029
	EP 267155	A3	19880720		
	R: AT, BE, CH, DE, ES, FR, GB, GR, IT, LI, LU, NL, SE				
	NO 8704489	A	19880505	NO 1987-4489	19871028
	FI 8704804	A	19880505	FI 1987-4804	19871102
	DD 275048	A5	19900110	DD 1987-308578	19871102
	DK 8705756	A	19880505	DK 1987-5756	19871103
	ZA 8708245	A	19880629	ZA 1987-8245	19871103
	HU 48611	A2	19880628	HU 1987-4930	19871103
	AU 8780655	A1	19880505	AU 1987-80655	19871104
	AU 606087	B2	19910131		
	JP 63130589	A2	19880602	JP 1987-277528	19871104
	US 4814346	A	19890321	US 1987-116737	19871104
PRAI	GB 1986-26344		19861104		
OS	MARPAT 109:149354				
GI					

L5 ANSWER 13 OF 19 CA COPYRIGHT 2000 ACS
 AN 103:33570 CA
 TI Inhibition of in vitro microsomal lipid peroxidation by isoflavonoids
 AU Jha, Hem Chandra; Von Recklinghausen, Gottfried; Zilliken, Fritz
 CS Inst. Physiol. Chem., Univ. Bonn, Bonn, D-5300/1, Fed. Rep. Ger.
 SO Biochem. Pharmacol. (1985), 34(9), 1367-9
 CODEN: BCPCA6; ISSN: 0006-2952
 DT Journal
 LA English
 GI

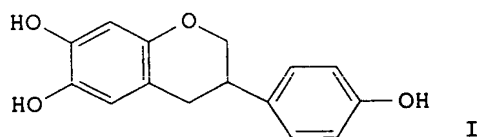


AB In a comparative study, the inhibition by naturally occurring isoflavones (with nucleus I) and their reduced derivs. (isoflavanones and isoflavans) of microsomal lipid peroxidn. induced by an Fe²⁺-ADP complex and NADPH was examd. The isoflavanones are more active than the parent isoflavones, and the isoflavans are by far the most potent inhibitors. In an in vitro test system, 6,7,4'-trihydroxy- and 6,7-dihydroxy-4'-methoxyisoflavans (concns. for half-maximal inhibition 1.3 and 1.1 .times. 10⁻⁶ mol/L, resp.) surpass the inhibitory effect of .alpha.-tocopherol, (+)-cyanidanol-3, and butylated hydroxyanisole. To establish a structure-activity relationship, a few more isoflavans have been included in the investigation.

IT 97148-44-2
 (lipid peroxidn. by microsome inhibition by)
 RN 97148-44-2 CA
 CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-phenyl- (9CI) (CA INDEX NAME)



L5 ANSWER 14 OF 19 CA COPYRIGHT 2000 ACS
 AN 102:55840 CA
 TI 6,7,4'-Trihydroxyisoflavan: a potent and selective inhibitor of
 5-lipoxygenase in human and porcine peripheral blood leukocytes
 AU Kuhl, P.; Shiloh, R.; Jha, H.; Murawski, U.; Zilliken, F.
 CS Inst. Physiol. Chem., Rheinischen Friedrich-Wilhelms-Univ., Bonn, D-5300,
 Fed. Rep. Ger.
 SO Prostaglandins (1984), 28(6), 783-804
 CODEN: PRGLBA; ISSN: 0090-6980
 DT Journal
 LA English
 GI

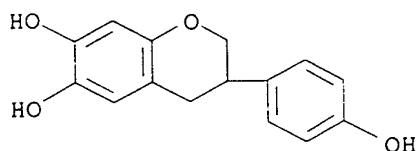


AB The effects of 6,7,4'-trihydroxyisoflavan (I) [94105-89-2] on human platelet 12-lipoxygenase [82391-43-3] and human and porcine polymorphonuclear leukocyte 5-lipoxygenase [80619-02-9] activities were examd. I inhibited 5-lipoxygenase more strongly than 12-lipoxygenase; its concn. for 50% inhibition (IC50) was 1.6 .mu.M for human and porcine 5-lipoxygenase and 22 .mu.M for human platelet 12-lipoxygenase. Inhibition of microsomal cyclooxygenase [39391-18-9] from ram seminal vesicles was exhibited at much higher concns. of I (IC50 = 200 .mu.M).

IT 94105-89-2
(lipoxygenase inhibition by, in blood platelet and leukocyte of human and swine)

RN 94105-89-2 CA

CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-hydroxyphenyl)- (9CI) (CA INDEX NAME)



L5 ANSWER 15 OF 19 CA COPYRIGHT 2000 ACS

AN 102:42728 CA

TI Antifungal activity of soybean and chickpea isoflavones and their reduced derivatives

AU Kraemer, Rainer Philipp; Hindorf, Holger; Jha, Hem Chandra; Kallage, Jutta; Zilliken, Fritz

CS Inst. Pflanzenkrankh., Univ. Bonn, Bonn, D-5300/1, Fed. Rep. Ger.

SO Phytochemistry (1984), 23(10), 2203-5
CODEN: PYTCAS; ISSN: 0031-9422

DT Journal

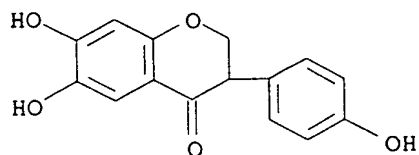
LA English

AB The fungicidal activity of the isoflavones from soybean (*Glycine max*) and chickpea (*Cicer arietinum*) was studied on 3 food- and forage-contaminating fungi, *Aspergillus ochraceus*, *Penicillium digitatum*, and *Fusarium culmorum*. The reduced derivs. of the corresponding isoflavones, the isoflavanones and isoflavans, were also included in the investigation. For the 1st time in a comparative study it was shown that isoflavones and isoflavanones are variable in their activity whereas the isoflavans are moderately active inhibitors of fungal growth.

IT 94105-87-0 94105-89-2
RL: BAC (Biological activity or effector, except adverse); BIOL (Biological study)
(antifungal activity of)

RN 94105-87-0 CA

CN 4H-1-Benzopyran-4-one, 2,3-dihydro-6,7-dihydroxy-3-(4-hydroxyphenyl)- (9CI) (CA INDEX NAME)



RN 94105-89-2 CA

CN 2H-1-Benzopyran-6,7-diol, 3,4-dihydro-3-(4-hydroxyphenyl)- (9CI) (CA INDEX NAME)

Effect of Soy-Derived Isoflavonoids on the Induced Growth of MCF-7 Cells by Estrogenic Environmental Chemicals

Surendra P. Verma and Barry R. Goldin

Abstract: Isoflavonoids are natural plant compounds and possess antitumorigenic properties. Many environmental chemicals have been found to be estrogenic and can enhance tumor growth in estrogen receptor-positive cells. In the present study, the effects of genistein, daidzein, biochanin A, formononetin, and equol on the proliferation of estrogen receptor-positive MCF-7 cells induced by synthetic chemicals (o-chlorophenyl)-1-(p-chlorophenyl)-2,2,2-trichloroethane (o,p'-DDT), 4-nonylphenol (4-NP), and 5-octylphenol (5-OP) found in the environment were investigated. Genistein, biochanin A, equol, and to some extent daidzein, but not formononetin, at $<10 \mu\text{M}$ can enhance the growth of MCF-7 cells in the absence of environmental chemicals. Formononetin was toxic to MCF-7 cells at the tested concentrations. The environmental chemicals 4-NP, 5-OP, and o,p'-DDT and the natural estrogen 17β -estradiol at 5, 5, and $10 \mu\text{M}$ and 5 nM, respectively, induced proliferation of MCF-7 cells. In the presence of isoflavonoids ($>25 \mu\text{M}$), the environmental chemical-induced cell proliferation was inhibited. Individually, genistein ($IC_{50} = 25\text{--}33 \mu\text{M}$) was the most potent inhibitor against the induced proliferation of MCF-7 cells of the isoflavonoids needed for a 50% suppression of growth induced by 4-NP, 5-OP, and o,p'-DDT. A mixture of isoflavonoids was the most potent inhibitor against the induced proliferation. Estrogen receptor-dependent and -independent pathways could be involved in the inhibitory actions of isoflavonoids. Because it is impossible to have a chemical-free environment, the in vitro data presented here are of practical importance to develop evolving dietary strategies and tactics against the adverse health effects of environmental chemicals.

Introduction

Certain pesticides and environmental contaminants such as 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane (DDT), 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2,2-trichloroethane (o,p'-DDT), 4-nonylphenol (4-NP), 5-octylphenol (5-OP), and polychlorinated biphenyls (PCB) show estrogenic activities (1-5). Similarly, some sewage and industrial waste

products and chemicals used in manufacturing plastics and detergents also have been found to be estrogenic (6,7). The estrogenic activities of the environmental chemicals o,p'-DDT, 4-NP, and 5-OP have been confirmed using estrogen receptor element-transfected cells (3,8,9). There are data showing that man-made environmental chemicals have modified estrogen-dependent biologic functions of various wildlife animal species (10,11). These findings have stimulated a growing public concern that environmental estrogenic chemicals may adversely affect human health and more specifically the estrogen-dependent functions (1,12). Environmental estrogenic compounds have been linked to increased incidence of breast and endometrial cancers (13-15). A recent report, however, comparing blood levels of PCB and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (the main metabolite of DDT) among women who had been diagnosed with breast cancer and matched healthy controls showed no difference in these levels (16). These data are in contrast to earlier studies, showing a link between exposure to DDT and PCB and increased incidence of breast tumors (13-15). It would be of interest to know whether the dietary intake of isoflavones was different between the controls and breast cancer patients, since the tumor-initiating and -promoting properties of the pesticides could be different depending on the isoflavonoid content of the diet (17).

It is difficult to have an estrogenic chemical-free environment, since many of these compounds have commercial value and are being used to manufacture products. Also, it is difficult to completely remove chemicals that have been released into the environment. Furthermore, there is a possibility that many more environmental chemicals that possess some estrogenic activity will be discovered. Because of these concerns and economic and health-related trade-offs, we previously suggested a dietetic strategy that could reduce the risk from estrogenic pesticides as assessed by the induced growth of estrogen receptor-positive MCF-7 human breast cancer cells (18).

The aim of the present study was to extend this strategy and to develop a cocktail of natural plant products, such as soy-derived isoflavonoids, that could inhibit the estrogenic effects of environmental chemicals on the estrogen recep-

tor-positive MCF-7 human breast cancer cells. The DDT isomer *o,p'*-DDT and the chemicals 4-NP and 5-OP were selected as the well-established estrogenic compounds (1-5). Although DDT and other chlorinated pesticides have been banned in the developed countries, their bioaccumulation in fat and blood is still found in people from all parts of the world (15,19-21) and thus is a continued risk. A recent report has indicated that during periods of lipolysis in fasting animals preloaded with the pesticide β -hexachlorocyclohexane the pesticide is released from fat cells (22). The released pesticide was able to stimulate the estrogen target tissues, suggesting a possible link between obesity and the progression of estrogen-dependent tumors (22). A recent study has added further support to this association by showing that the incidence of breast cancer and mortality from the disease doubled in postmenopausal women who had gained >25 kg in weight since they were 18 years of age (23).

Isoflavonoids are found in high concentrations in soybeans. The two most common isoflavonoids are genistein and daidzein, which have been shown to have many beneficial clinical and antitumorigenic properties (24-28). We reported previously that curcumin and genistein individually or in combination can inhibit estrogenic pesticide-induced growth of estrogen receptor-positive MCF-7 human breast cancer cells (18). Here we describe the effects of genistein, daidzein, biochanin A, formononetin, and equol, individually and in combination, on the proliferation of estrogen receptor-positive MCF-7 human breast cancer cells induced by *o,p'*-DDT, 4-NP, and 5-OP. Formononetin is a precursor of daidzein and biochanin A is a precursor of genistein, whereas equol is a bacterial metabolite of daidzein formed in the intestinal tract (17). These soy-derived products (SDP) can inhibit the induced proliferation of estrogen receptor-positive human breast cells by environmental chemicals and pesticides and also the proliferation of estrogen receptor-negative MDA-MB-231 human breast cancer cells.

Materials and Methods

Chemicals

Stock solutions of *o,p'*-DDT (Ultra Scientific, Kingston, RI), 4-NP and 5-OP (Aldrich Chemical, Milwaukee, WI), 17β -estradiol (Sigma Chemical, St. Louis, MO), and genistein, daidzein, biochanin A, formononetin, and equol (Indofine Chemical, Belle Mead, NJ) were prepared by dissolving the compounds in dimethyl sulfoxide. All stock solutions of compounds were diluted with phenol red-free tissue culture medium supplemented with 5% dextran-coated charcoal-treated human serum (HuDCC; Sigma Chemical) and 100 U/ml of penicillin-streptomycin to an appropriate concentration (test medium). The control medium contained $\leq 1\%$ ethanol or dimethyl sulfoxide or their 1:1 mixture.

Cell Culture and Growth Procedure

Estrogen receptor-positive human breast cancer MCF-7 and estrogen receptor-negative MDA-MB-231 human breast cancer cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and 5 ml of 10,000 U/500 ml medium of penicillin-streptomycin (GIBCO BRL) in an incubator maintained at 5% CO_2 -95% air and 100% humidity at 37°C.

Before each experiment with MCF-7 cells, phenol red maintenance medium was replaced with phenol red-free medium for 24 hours. The phenol red-free medium was then replaced with phenol red-free RPMI 1640 medium supplemented with 5% HuDCC (growth factor stripped) for 48 hours. Cells were detached by adding 2-3 ml of a 4% trypsin solution. After 15 minutes, cells were harvested and washed with Ca-Mg-free phosphate-buffered saline. Washed cells were suspended in phenol red-free RPMI 1640 medium supplemented with 5% HuDCC and antibiotics and were gently agitated by passing up and down in a pipette to obtain a single cell suspension. Cells were counted by a Coulter counter. Cells ($2-3 \times 10^5/\text{ml}$) were plated in a 12-well culture plate (2 ml/well) and allowed to attach for 48 hours.

After 48 hours, medium was removed and replaced by a test medium prepared as described above containing the appropriate concentrations of environmental chemicals and isoflavones. Control cells were incubated with a phenol red-free medium containing 5% HuDCC and an equivalent amount of the solvent(s) without the test material. The solvent concentration did not exceed 1%, and this concentration does not appreciably alter cell growth. The cell growth was estimated on Day 6 by using the tetrazolium (MTT) assay, as has been reported previously (18). The absorbance at 540 nm was used as a measure of the cell density of live cells. The percent proliferation was calculated as follows: percent proliferation = (cell density with test compound/cell density of control) $\times 100$.

All data were normalized to the 100% cell proliferation induced by the respective environmental estrogens. Results are an average of at least three separate experiments, and in each experiment, each data point was carried out in triplicate.

The following procedure was used to determine the growth enhancement or toxicity of SDP on the growth of MCF-7 cells. Cells growing in phenol red-free RPMI 1640 medium supplemented with 2% FCS were plated in 12-well plates. After 24 hours the medium was replaced by phenol red-free RPMI 1640 medium supplemented with 2% FCS containing SDP of varying concentrations. Cells were allowed to grow for four days, and on Day 4 the growth was estimated by the MTT assay by using the equation shown above. The cell growth in control medium was normalized to 100%.

Estrogen receptor-negative MDA-MB-231 cells (2×10^5 in each well) were plated in 12-well plates and allowed to attach for 24 hours. The medium was replaced with the medium containing appropriate concentrations of soy prod-

ucts. Cell densities were estimated on Day 4 by using the MTT assay. Growth of control and treated cells was calculated using the above equation, and data were normalized to 100%.

Results

Effect of SDP on Cell Growth

Low levels of genistein, daidzein, biochanin A, and equol enhanced the growth of MCF-7 cells (Figure 1). All four compounds enhanced the proliferation of MCF-7 cells when present at $<10 \mu\text{M}$. Maximum proliferation of MCF-7 cells was found when biochanin A and equol were present at $\leq 10 \mu\text{M}$. At $50 \mu\text{M}$, biochanin A and equol do not increase cell growth or cause cell toxicity. Genistein and daidzein at $\leq 5 \mu\text{M}$ enhance cell growth by about 45% and 20%, respectively. With $10\text{--}50 \mu\text{M}$ genistein, daidzein, biochanin A, and equol, there is negligible toxicity (within experimental error, $\pm 5\text{--}7\%$) to MCF-7 cells. All the compounds tested were toxic to MCF-7 cells at $100 \mu\text{M}$. Formononetin does not enhance the proliferation of MCF-7 cells. However, formononetin is toxic to MCF-7 cells at $50 \mu\text{M}$. The concentrations of formononetin, biochanin A, and genistein required to inhibit growth of MCF-7 cells by 50% (IC_{50}) are 30, 75, and $75 \mu\text{M}$, respectively. Daidzein and equol are comparatively less toxic, with an $\text{IC}_{50} > 100 \mu\text{M}$.

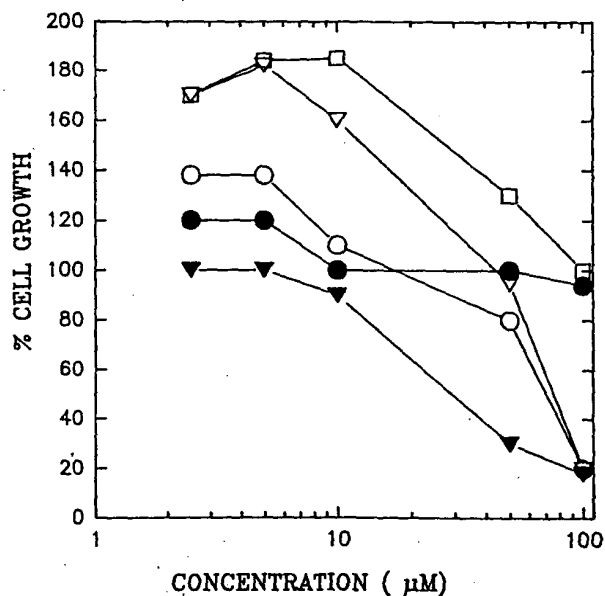


Figure 1. Effect of isoflavonoid concentration on proliferation of MCF-7 cells. Cells ($2 \times 10^5/\text{well}$) were seeded in 12-well plates in dye-free RPMI 1640 supplemented with 2% fetal calf serum for 24 h. Medium was replaced by test medium containing an appropriate concentration of isoflavonoids. Cell growth was measured on Day 4 using tetrazolium (MTT) assay. Growth of cells in control medium without isoflavonoids was normalized to 100%. Open circles, genistein; filled circles, daidzein; open triangles, biochanin A; filled triangles, formononetin; open squares, equol.

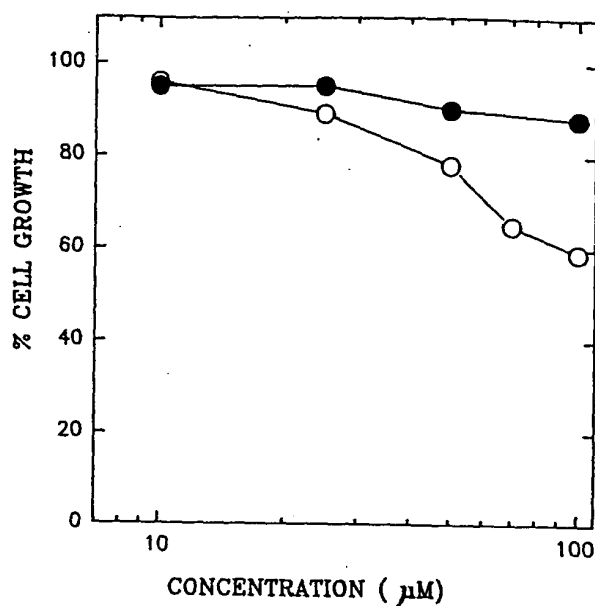


Figure 2. Effect of genistein (open circles) and daidzein (filled circles) concentration on proliferation of MDA-MB-231 cells. Cells ($1 \times 10^5/\text{well}$) were seeded in 12-well plates in RPMI 1640 supplemented with 2% FCS for 24 h. Medium was replaced by test medium containing an appropriate concentration of genistein and daidzein. Cell growth was measured on Day 4 using MTT assay. Growth of cells in control medium without isoflavonoids was normalized to 100%.

Genistein was ineffective in enhancing the growth of estrogen receptor-negative MDA-MB-231 human breast tumor cells at lower concentrations (Figure 2). At $>50 \mu\text{M}$, genistein reduces the growth of MDA-MB-231 cells. Daidzein did not alter the growth of MDA-MB-231 cells.

Effects of Isoflavonoids Against the Induction of MCF-7 Cell Growth by 4-NP and 5-OP

The percent proliferation of *o,p'*-DDT ($10 \mu\text{M}$), 4-NP ($5 \mu\text{M}$), and 5-OP ($5 \mu\text{M}$) was 100, 93, and 93, respectively (error $\pm 5\text{--}7\%$), relative to 5 nM 17β -estradiol for the estrogen receptor-positive MCF-7 cell line. The proliferation potencies of these compounds are within the range of the previously published data (2,8,9,29). Concentrations of $\geq 15 \mu\text{M}$ were toxic for 4-NP and 5-OP.

The concentration-dependent effects of the isoflavonoid test compounds on 4-NP-induced proliferation of MCF-7 cells are shown in Figure 3. The 4-NP-induced cell proliferation was normalized to 100%; all other data presented in Figure 3 are with respect to this normalized value. When cells were incubated in the test medium containing various concentrations of isoflavonoids, the induction of cell proliferation by $5 \mu\text{M}$ 4-NP was significantly suppressed. The IC_{50} values for equol, genistein, formononetin, and biochanin A are 27, 30, 36, and $40 \mu\text{M}$, respectively. Daidzein does not inhibit 4-NP-induced cell proliferation at $<50 \mu\text{M}$ (Figure 3).

In Figure 4 the effects of isoflavonoids on the 5-OP-induced proliferation of MCF-7 cells are shown. The cell proliferation induced by $5 \mu\text{M}$ 5-OP has been normalized

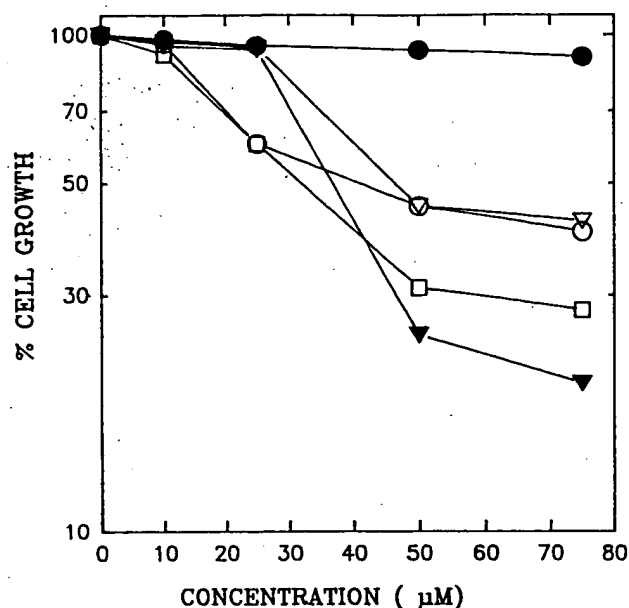


Figure 3. Effect of isoflavonoid concentration on proliferation of MCF-7 cells induced by 4-nonylphenol (5 μ M). Cells (2×10^5 /well) were seeded in 12-well plates in dye-free RPMI 1640 supplemented with 5% dextran-coated charcoal-treated human serum for 24 h. Medium was replaced by test medium containing an appropriate concentration of isoflavonoids. Cell growth was measured on Day 6 using MTT assay. Control wells contained an appropriate amount of DMSO without flavonoids. Induced cell growth by 4-nonylphenol has been normalized to 100%. See Figure 1 legend for explanation of symbols.

to 100%, and other data are presented with respect to this value. The IC_{50} values for genistein and biochanin A are about 33 μ M. The IC_{50} values for daidzein, formononetin, and equol cannot be accurately obtained from Figure 4. However, the induced growth inhibition by these compounds is weak, and the IC_{50} is >80 μ M.

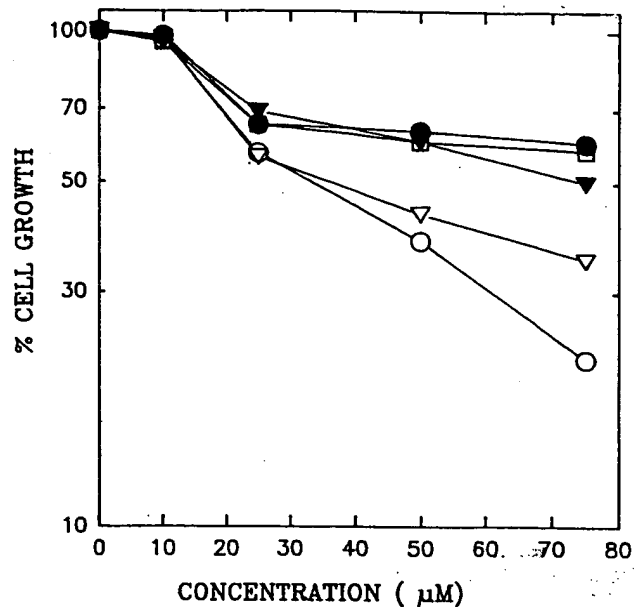


Figure 4. Effect of isoflavonoid concentration on proliferation of MCF-7 cells induced by 5-octylphenol (5 μ M). Cells (2×10^5 /well) were seeded in 12-well plates in dye-free RPMI 1640 supplemented with 5% dextran-coated charcoal-treated human serum for 24 h. Medium was replaced by test medium containing an appropriate concentration of flavonoids. Cell growth was measured on Day 6 using MTT assay. Control wells contained an appropriate amount of DMSO without isoflavonoids. Induced cell growth by 5-octylphenol has been normalized to 100%. See Figure 1 legend for explanation of symbols.

Mixtures of isoflavonoids suppress the 4-NP- and 5-OP-induced cell proliferation and cause cell death (Table 1). At 25 μ M genistein, daidzein, biochanin A, and formononetin, the 4-NP- and 5-OP-induced cell proliferation is reduced to 24% and 32%, respectively. A mixture of biochanin A and formononetin at 50 μ M each kills 15% of the cells. Similar

Table 1. Effect of Isoflavonoids on Percent Proliferation of MCF-7 Cells Induced by Environmental Chemicals and Effect of Environmental Chemicals and Isoflavonoids on Growth of MDA-MB-231 Cells^{a,b}

Test Compounds	Concentration, μ M	%Proliferation
<i>MCF-7 cells</i>		
17 β -Estradiol	0.005	100
<i>o,p'</i> -DDT	10	100
4-NP	5	93
5-OP	5	93
<i>o,p'</i> -DDT + Gen + Bio	10 + 25 + 25	26
<i>o,p'</i> -DDT + Gen + For	10 + 25 + 25	23
<i>o,p'</i> -DDT + Gen + Daid + Bio + For	10 + 25 + 25 + 25 + 25	10
4-NP + Gen + Daid + Bio + For	5 + 25 + 25 + 25 + 25	24
5-OP + Gen + Daid + Bio + For	5 + 25 + 25 + 25 + 25	32
<i>MDA-MB-231 cells</i>		
<i>o,p'</i> -DDT	10	67
4-NP	5	70
5-OP	5	72
Gen + Daid	25 + 25	70
Gen + Daid	50 + 50	63

a: Calculated induced growth of MCF-7 cells by 5 nM 17 β -estradiol has been normalized to 100%. The rest of the values are normalized to 100% growth of 17 β -estradiol. Average values of ≥ 3 separate experiments are presented. Growth of MDA-MB-231 cells in control medium without test compounds has been normalized to 100% and taken as a full growth of cells.

b: Abbreviations are as follows: *o,p'*-DDT, 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane; 4-NP, 4-nonylphenol; 5-OP, 5-octylphenol; Gen, genistein; Daid, daidzein; Bio, biochanin A; For, formononetin.

killing of cells is also observed for mixtures containing genistein and biochanin A (50 μM each) and genistein and equol (50 μM each).

Effects of Isoflavonoids on the *o,p'*-DDT-Induced Cell Proliferation

In Figure 5 the data are presented for the inhibitory action of genistein, daidzein, biochanin A, formononetin, and equol toward *o,p'*-DDT-induced proliferation of MCF-7 cells. The *o,p'*-DDT-induced cell proliferation has been normalized to 100%. The IC_{50} values for equol, genistein, formononetin, biochanin A, and daidzein are 25, 26, 53, 92, and >100 μM , respectively. These data show that biochanin A and daidzein are the weakest inhibitors of estrogenic pesticide-induced cell proliferation.

The combined inhibitory effects of these compounds toward the *o,p'*-DDT-induced cell proliferation are shown in Table 1. A mixture of genistein-biochanin A and genistein-formononetin at 25 μM each can inhibit the *o,p'*-DDT-induced proliferation of MCF-7 cells to 26% and 23% of the normalized control, respectively. A genistein-biochanin A-formononetin mixture at 25 μM each inhibits the induced proliferation to 10% of the proliferation noted for these cells in the *o,p'*-DDT and in the absence of the mixture of these three soybean products.

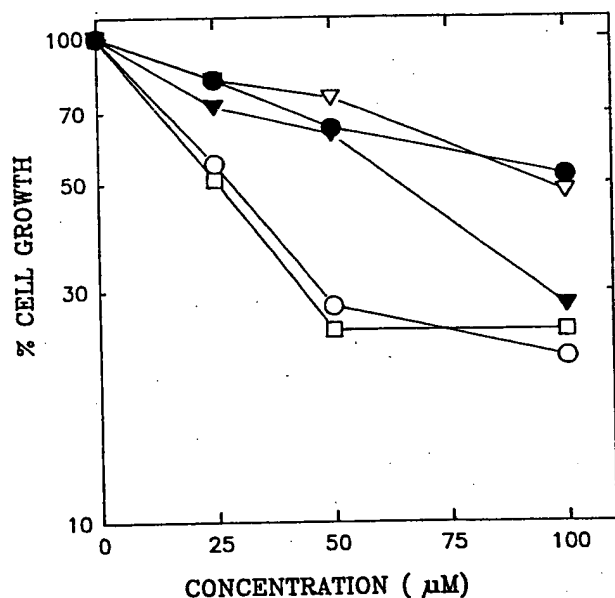


Figure 5. Effect of isoflavonoid concentration on proliferation of MCF-7 cells induced by 10 μM 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (*o,p'*-DDT). Cells (2×10^5 /well) were seeded in 12-well plates in dye-free RPMI 1640 supplemented with 5% dextran-coated charcoal-treated human serum for 24 h. Medium was replaced by test medium containing an appropriate concentration of isoflavonoids. Cell growth was measured on Day 6 using MTT assay. Control wells contained an appropriate amount of DMSO without isoflavonoids. Induced cell growth by *o,p'*-DDT has been normalized to 100%. See Figure 1 legend for explanation of symbols.

Discussion and General Comments

Figures 1 and 2 show that genistein, daidzein, biochanin A, and equol, but not formononetin, at <10 μM can increase the proliferation of estrogen receptor-positive MCF-7 but not estrogen receptor-negative MDA-MB-231 cells. The growth-stimulatory effect of genistein reported here is comparable to the results of Balabhadrapathruni and co-workers (30). These investigators showed that 10 μM genistein significantly increased DNA synthesis in MCF-7 cells. However, the cytotoxic concentration of 25 μM genistein that caused a 50% reduction in DNA synthesis is significantly lower than that observed in this study ($\text{IC}_{50} = 60$ μM). So and colleagues (31) also reported an enhancement in MCF-7 cell growth induced by genistein, but they used a higher concentration (5 $\mu\text{g}/\text{ml}$ or 18 μM). Discrepancy in the genistein concentration that stimulates growth and causes cytotoxicity still exists. Peterson and Barnes (32) reported an IC_{50} of 12.5 $\mu\text{g}/\text{ml}$ (or 39.5 μM) of genistein for MCF-7 cells. Pagliacci and associates (33) showed that in the presence of 30 μM genistein the growth of MCF-7 cells increased to about 150% when measured by MTT assay. In contrast, 30 μM genistein has been found to be toxic when measured with a hemocytometer (direct cell counting). The reported genistein concentration discrepancy may probably arise from the type of assays used, or MCF-7 cells may have different clones in these experiments. The concentrations of isoflavones used in the present study are higher and probably are in the pharmacological range. Although higher concentrations of isoflavones cannot be achieved by eating foods rich in isoflavones, there are data showing a linear relationship between the oral dose of isoflavonoids and urinary excretion of these compounds (34). Therefore, a semipure powdered isoflavonoid preparation in tablet or capsule form could be used to reach pharmacological concentrations.

These data suggest that the enhancement of MCF-7 cell proliferation caused by genistein, equol, and biochanin A may be mediated by an estrogen receptor-dependent pathway, since genistein and daidzein at 10 μM have a negligible effect on the proliferation of estrogen receptor-negative cells (MDA-MB-231). However, isoflavonoids did not increase cell proliferation of estrogen receptor-positive T47D cells (unpublished data). The inhibition by 50 μM genistein is substantial. The exact explanation for the enhancement in proliferation of MCF-7, but not of T47D, cells by isoflavones and their metabolites is not known. It may, however, be stated that these compounds may also enhance the proliferation of estrogen receptor-positive cells through an estrogen receptor-negative-independent pathway and that this pathway is not sensitive to isoflavones in T47D cells. Tamoxifen, a weak estrogen, at 1–5 μM , has enhanced the growth of estrogen receptor-negative MDA-MB-231 cells. The latter data suggest that some weak estrogens can enhance cell growth through estrogen-independent pathways.

At 10–25 μM , isoflavonoids have been found to be non-toxic to MCF-7 cells. Genistein and biochanin A are slightly

toxic at 50 μM . Formononetin is highly toxic in this concentration range. Equol and daidzein are less toxic toward MCF-7 cells. The mechanisms involved in the inhibition of cell proliferation or toxicity by isoflavones and their metabolites are also not clear. It is possible that the proliferation of estrogen receptor-positive cells is mediated through estrogenic and unidentified nonestrogenic pathway(s). One of the estrogen-independent pathways may involve an alteration in membrane fluidity (35,36). The change in membrane fluidity could be transient. It has been reported that membrane fluidity may deregulate internal Ca^{2+} concentration (37). Deregulation in internal Ca^{2+} concentration can cause cell proliferation or cell death, depending on the increasing or decreasing cytoplasmic Ca^{2+} concentration (38). These membrane-related events can modulate the activities of lipid- and Ca^{2+} -dependent protein kinase C, since the insertion of protein kinase C into membrane lipids is essential for its activation (39). The activation of protein kinase C modulates several cellular functions, including cell growth and cell death. Tamoxifen at 10 μM can alter the fluidity of MCF-7 plasma membrane lipids as detected by Raman spectroscopy (unpublished results). It has been recently reported that tamoxifen at higher concentration ($>1 \mu\text{M}$) elevates internal Ca^{2+} levels, leading to the death of MCF-7 cells (40). We also found that 10 μM tamoxifen kills about 30% of MCF-7 cells (data not shown). In view of these data, it is tempting to speculate that the inhibition of cell proliferation (cells growing in 2% FCS) by isoflavonoids at higher concentrations (50–100 μM) could be a result of altering the membrane fluidity. Another mechanism proposed for stimulatory and cytotoxic action suggested by Balabhadrapathruni and co-workers (30) involves a genistein-induced increase and decrease in the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase at 10 and 25 μM , respectively.

The environmental chemicals 4-NP and 5-OP and the DDT pesticide isomer *o,p'*-DDT have been found to induce proliferation of MCF-7 at $\leq 10 \mu\text{M}$. Several authors have reported that DDT and its isomer *o,p'*-DDT functions as an estrogen at $\leq 10 \mu\text{M}$. DDT has been found at 6 nM–1.7 μM in the sera of the studied population (41). DDT and its metabolite dichlorodiphenyldichloroethylene (DDE) can accumulate 1,000-fold higher in the mammary adipose tissue (41). The concentration of DDE was 2.2 $\mu\text{g/g}$ of lipid in the human breast cancer cells (13). Although the exact comparison of the concentration of DDT between the *in vitro* and *in vivo* studies and the resultant estrogenic effect seems to be impractical, the time of incubation in *in vivo* systems is extremely long (months to years), whereas the DDT effect can be observed in a few days in *in vitro* experiments. However, the measured concentration of DDT reported in human breast cells (13) is approximately 500 times lower (as calculated below) than that generally used in *in vitro* experiments to determine the estrogenic effect. On the basis of some assumptions, the volume of a single cell is about $2.85 \times 10^{-12} \text{ cm}^3$ (volume = $4/3 \times \pi \times r^3$, where r , the radius, can be taken as 0.0013 cm). One milliliter contains approximately 5×10^8 cells. With an average density of 1.1, the

total mass of 1 ml of cells will be approximately 6.6 mg. The average mass of total lipids in 1 ml of cells may be about 2.64 mg (40% of total mass). In view of the measured values of DDT as 2.2 $\mu\text{g/g}$ lipid by Falck and others (13), 1 ml of breast tumor cells should contain about 0.006 μg of DDT, which is about 500 times lower than that used in the present studies. Even the higher concentration of *o,p'*-DDT (10 μM) used in *in vitro* studies was not toxic, but stimulatory, to the growth of MCF-7 cells.

In contrast, these environmental chemicals have been found to be toxic to the estrogen receptor-negative MDA-MB-231 cells at 1–10 μM (Table 1). These data are in accordance with the previously published results suggesting that 4-NP, 5-OP, and *o,p'*-DDT induce proliferation in estrogen receptor-positive cells through estrogen-mediated pathways (2,8,9,29). Isoflavones when present at $>25 \mu\text{M}$ can inhibit the proliferation induced by 4-NP, 5-OP, and *o,p'*-DDT. As shown in Table 1, a mixture of isoflavones and their metabolites is a more efficient inhibitor of estrogen-like chemical-induced cell growth.

The order of inhibition by isoflavones was different for proliferation induced by 4-NP, 5-OP, or *o,p'*-DDT. Data presented in Figures 3–5 indicate that 50 μM genistein was the most potent inhibitor (within the experimental error, $\pm 5\%$) of the proliferation of MCF-7 cells induced by 4-NP, 5-OP, and *o,p'*-DDT. Daidzein was the poorest inhibitor among the three chemicals tested.

The mechanisms for inhibition of this induced proliferation by isoflavonoids are complex and have not been fully explored. By use of estrogen-responsive element (ERE)-transfected nonestrogen receptor cells, it has been demonstrated by several researchers that *o,p'*-DDT induces proliferation through the interaction with the ERE (8,9). Similarly, it also has been demonstrated that octylphenol and nonylphenol mimic this action (3). In view of this mechanism, the inhibition of induced proliferation by isoflavones could be through 1) blocking the pathway or cellular events that lead to the interaction of environmental chemicals with ERE or 2) directly blocking the interaction between ERE and environmental estrogens. Protein kinases play a key role in regulating the cell proliferation, and genistein is now well recognized for inhibiting properties of various kinds of protein kinases (e.g., protein kinase C, tyrosine kinase, mitogen-activated protein kinase) (42–44). Because protein kinases are involved in the phosphorylation of estrogen and progesterone receptors and because phosphorylated receptors become activated, genistein might be directly interfering with the receptor phosphorylation process. Also, there is a possibility that induced cell growth inhibition and death resulting from blocking the activities of protein kinases might be transiently triggered on alteration of membrane fluidity by isoflavones. It is possible, therefore, that induced proliferation of estrogen receptor-positive cells and inhibition of growth could occur through estrogen receptor-dependent and -independent pathways. The activities of other isoflavonoids used in this study toward protein kinases are not known. The other receptor-independent pathway for inhibitory actions by isoflavones

against the induced cell growth may involve inhibition of DNA topoisomerase II (45).

Pesticides and environmental chemicals discovered to be estrogenic are still widespread in our ecosystem. Most of these compounds are lipid soluble, resist metabolizing, and are chemically stable. These properties may allow them to accumulate in humans or animals for a longer period of time. Furthermore, it is almost impossible to eliminate these compounds from the environment. Because identified and unrecognized environmental estrogens may remain in the environment, food, water, animals, or humans, the dietary strategies suggested previously (18) and supported by the *in vitro* data presented in this study may be of practical importance in preventing the adverse health effects of these environmental estrogens.

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The New Biology of Steroid Hormones

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Lignans, Isoflavones, Sex Hormone Metabolism and Breast Cancer

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Introduction

Breast, colon and prostate cancer and coronary heart disease incidence and mortality are high in the Western world compared to countries in Asia and some East European countries (16,36,52). A diet rich in fat and protein but poor in fiber and complex carbohydrates, particularly whole-grain products is called a Western diet. In fact numerous migrant studies support the view that this diet is one of the main factors causing the high incidence of the s.c. Western diseases (30,34,44,51,60,61,64). Because many of these diseases are hormone-dependent cancers it has been postulated, that the Western diet compared to the vegetarian or semivegetarian diet in some developing and Asian countries by some biochemical or other mechanisms may alter hormone production, metabolism or action at the cellular level. Since more than 10 years the interest of our group has been focussed on these mechanisms. In this connection we will discuss the possible role of some new diphenolic compounds, the lignans and isoflavonic phytoestrogens, in the regulation of hormone levels and the effects of these compounds at the cellular level.

Lignans and isoflavones in the human organism

About ten years ago two cyclically occurring unknown compounds, now called enterolactone and enterodiol (58), were detected in the urine of the female vervet monkey (56) and women (54,57) and subsequently identified (59,62). They were shown to be diphenols with lignan structure lacking the *para*-oxygen substitution and differing in this way from plant lignans. They have also been found in men and children and in other biological materials and in some other animals (review and structures in 55). Recently four plant lignans, matairesinol (21), lariciresinol, isolariciresinol and secoisolariciresinol (22) were identified in human urine. Two 7'-hydroxylated compounds have also been detected and tentatively identified as 7'-hydroxymatairesinol and 7'-hydroxyenterolactone (unpublished).

The isoflavonic-phytoestrogens are heterocyclic phenols with a close similarity in structure to estrogens and their diphenolic character makes them also similar to lig-

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nans. They occur in numerous plants and many studies have shown that they have hormonal effects in animals (review in 48), the most important being the s.c. "clover disease" (25). The compound responsible for the disease is equol (7-hydroxy-3-[4-hydroxyphenyl]chroman) (see 37), formed by the ruminal bacteria from formononetin (45) present in ingested clover. Another group of similar compounds are the coumestans. The literature on the occurrence and metabolism of the phytoestrogens in animals (48) and in man (55) has been reviewed. The following phytoestrogens have been identified or detected in human urine for the first time in this laboratory: Formononetin, methylcoumestrol, daidzein, dihydrodaidzein, O-desmethylangolensin, genistein, and 3',7-dihydroxyisoflavone (21-23,55). Equol was identified independently in two laboratories (8,18).

Formation and metabolism of lignans and isoflavones in man

It has now been demonstrated that the mammalian lignans enterolactone and enterodiol are formed from precursors, such as the plant lignans matairesinol and secoisolaricresinol, which are consumed and then structurally modified by intestinal bacteria (55). Equol and O-desmethylangolensin are most likely formed by intestinal bacterial action from formononetin and daidzein present in food stuffs like soy products (9,20). The metabolism of isoflavones in man seems to be similar to that in sheep. These compounds are also present in cow milk (5) formed from e.g. formononetin in clover by intestinal bacteria in the gastrointestinal tract of the cow (48), and may therefore be consumed by human subjects as such. Because of the close association of lignan excretion with fiber intake (1,7,12) it is likely that the plant lignans are localized close to the outer fiber-containing layers of the grain containing phytin, polyphenols, enzyme inhibitors and other compounds usually regarded as antinutritional factors (27). Modern milling techniques usually eliminates this fraction, which does not, with some exceptions, anymore occur in the products supplied to the market for consumption.

Biological effects of lignans and isoflavonic phytoestrogens

The lignans enterolactone, enterodiol and matairesinol and the isoflavonic phytoestrogens daidzein, equol, O-desmethylangolensin and genistein have all weak estrogenic activity, but antiestrogenic activities have also been described (reviews in 2,3,55). Many plant lignans have been shown to have anticarcinogenic, antiviral, bactericidal and fungicidal activities. In collaboration with Dr Larry Vickery (Irvine, California) it was shown that enterolactone is a moderate inhibitor of placental aromatase and competes with the natural substrate androstenedione for the enzyme. Other experiments show that these diphenols are very readily transferred from cell culture media into the cells and that they may inhibit cancer cell growth. Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast cancer cell line, ZR-75-1, were reported (31) and also inhibitory effects on mitogen-induced proliferation of human peripheral blood lymphocytes (32). Genistein, one isoflavonic compound identified by us in human urine is a specific inhibitor of tyrosine-specific protein kinases (15,43,46,63). Protein-tyrosine kinase activity is associated with cellular receptors for many growth factors (EGF, insulin, IGF-1, PDGF and CSF-1), suggesting that the enzyme plays a role for cell proliferation and transformation. The enzyme has also been associated with oncogenic products of the retroviral src gene family and is correlated with the ability of retrovirus to transform cells (literature in 15,43,46,63).

In collaborative studies with Dr Jim Clark et al. we have found that several plant and mammalian lignans and isoflavones compete with estradiol (E2) for the rat uterine nuclear estrogen type II binding site (unpublished results). These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth (39,42). It was found that some flavonoids like luteolin, quercetin and p-larigenin inhibit E2 binding to this receptor and growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus (41). The structure of these compounds are very similar to those of the isoflavones. The most effective with regard to type II site binding of the diphenolic compounds found and measured by us in human urine seem to be the isoflavones daidzein and equol, but also some lignans like matairesinol, isolaricresinol and enterolactone show competition. Later Clark et al. identified an endogenous inhibitor of the nuclear type II binding site, methyl-p-hydroxyphenyllactate (40). It was postulated that uncontrolled growth and proliferation of malignant cells is directly related not only to the permanent stimulation of nuclear type II binding sites by estrogens or other compounds, but also to very low to nonmeasurable levels of the competitive inhibitor methyl-p-hydroxyphenyllactate (40). In our opinion it seems that probably many of these phenolic compounds may have a synergistic action as it is unlikely, because of close structural similarities, that only one of them inhibits cell growth.

Of the isoflavones the strongest estrogens are equol and genistein, but they are still very weak estrogens compared to estradiol and estrone. At present we can measure 3 lignans and 4 isoflavones by isotope dilution mass spectrometry in the selected ion monitoring mode using deuterated internal standards. The lignans and isoflavonic phytoestrogens are normal constituents of human urine and are excreted in large amounts particularly by vegetarians (both lignans and phytoestrogens) (6,8,12,13), by subjects consuming large amounts of whole-grain products, vegetables and berries (lignans) (12), and by the Japanese consuming traditional Japanese diet (mainly isoflavones), due to intake of soy products (9,12). In omnivorous Finnish subjects the excretion of genistein, the specific inhibitor of protein tyrosine kinase, was found to be between 0.01 and 1.5 $\mu\text{mol}/24\text{ h}$ (usually 1-4 times that of daidzein). When investigating a few Japanese men, women and children consuming a traditional diet the excretion was very high ranging from 1-15 $\mu\text{mol}/24\text{ h}$, about 1.5 - 3 times higher than that of daidzein (in collaboration with H. Honjo et al.) and 100-1000 times higher than the excretion of individual endogenous estrogens. As mentioned above, daidzein shows antiproliferative activity with regard to BC cells (31). Particularly low excretion of lignans and isoflavones has been observed in BC patients and in subjects consuming a low-fiber diet, especially a diet low in whole-grain products and beans (6,8,12,13).

Recently, we suggested that the lignans and isoflavones, perhaps together with other similar compounds, stimulate SHBG (sex hormone binding globulin) synthesis in the liver and in this way reduce the biological effects of sex hormones (3,12,13). An increase in SHBG results in lowering of % free testosterone (%FT) and % free estradiol (%FE2) and reduction of both the albumin-bound and the free fraction of the sex hormones. This reduces the metabolic clearance rate (MCR) of the steroids and reduces in this way their biological activity. Results in Finnish women shows that fiber-rich food increases total lignan excretion. Indeed, total fiber intake, total fiber intake/kg body weight and grain fiber intake/kg body weight correlate positively with urinary excretion of total lignans and isoflavones (12,13). The excretion of the two groups of compounds and also enterolactone alone in both pre- and postmenopausal (Fig. 1) Finnish women correlate positively with plasma

SHBG and negatively with plasma %FE2 and %FT (12,13 and unpublished results). We therefore suggest that these positive associations between urinary lignan and phytoestrogen excretion and SHBG are due to stimulation of SHBG synthesis by these week estrogens entering the portal circulation in very high amounts. This also would explain the higher SHBG values seen in vegetarians (11,13,17).

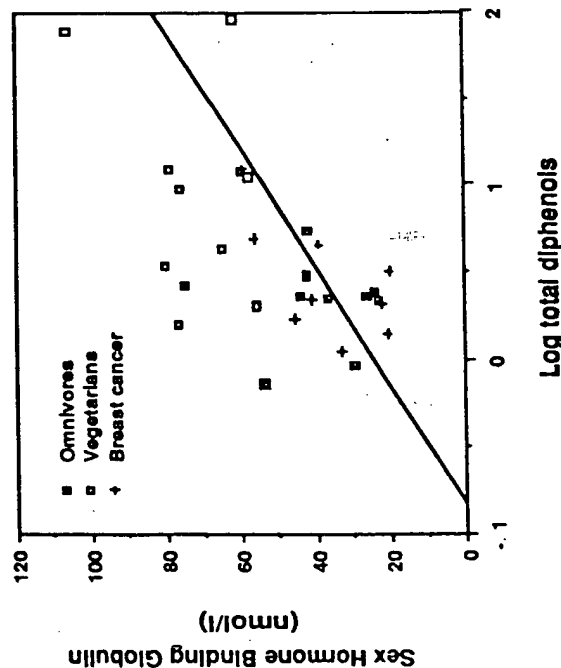


FIG. 1., Correlation between urinary excretion of diphenols (sum of enterolactone, enterodiol, daidzein, equol and O-desmethylangolensin) (logarithmic values) and plasma SHBG in postmenopausal Finnish omnivorous and vegetarian women and women with breast cancer.

Lignans and isoflavones, and breast cancer

In three case-control (26,38,66) and in an epidemiological study (35) it was shown that high fiber and high carbohydrate intake, respectively, are associated with decreased risk of BC. In another case-control study particularly fiber from grains consumed during adolescence reduced the risk both in young and old women (49). These observations are in agreement with the results of studies in postmenopausal women in Boston (11) and in premenopausal women in Helsinki (10) showing that the main and in fact the only really significant difference between the diets of the BC patients and the omnivorous and vegetarian control women was a low intake of grain products and fiber. If the diets of the Boston and Finnish women studied by us are compared, the main difference is also in the grain and grain fiber intake, being much higher in the Helsinki women with a lower risk for BC than in the Boston women. The fat to grain fiber ratio (g/g) was 16.4 in the old

Boston BC women and only 10.2 in the young Finnish BC women and the corresponding values for the omnivores were 15.1 and 8.2, respectively. The Boston and Helsinki vegetarians had total fat/grain fiber ratios of 7.1 and 6.3, respectively.

The diet in Finnish rural areas, where BC incidence is lower than in Helsinki, differs from the American one even more with regard to its relatively high content of complex carbohydrates mainly from whole-grain products and starchy vegetables, the fat content being similar but deriving more from milk products than from meat (50,53 and own observations). Particularly rye bread and other whole-grain products increase the excretion of urinary lignans by the Finns. The finding of very low urinary lignan excretion in the BC subjects living in Boston (8), later found to have very low intake of grain and grain fiber (11), and of lower excretion also in the young BC women in Helsinki (13) support the view that the lignans may be protective with regard to BC. However, in Helsinki the differences between the young omnivorous, vegetarian and BC groups were relatively small, because the grain intake was comparably high in all groups, which is typical for the original Finnish diet. It should be mentioned that the intake of wheat germ and bran do not at all cause increases in urinary lignan excretion in human subjects (own observations), and fiber-free wheat bread products have no or only very small influence on lignan excretion. Only grain products which have been made from milling of whole grain, without separating the different components and mixing them again (R. Korpela and H. Adlercreutz, to be published) seem to significantly increase lignan excretion in Finnish women. This is because during modern milling of the grain, trying to eliminate so-called antinutritional factors (27), simultaneously also the diphenolic plant lignans seem to be eliminated. There are indications that also berries, fruits and various seeds (12,19,55) increase lignan excretion. Of some grain products, rye meal seems to result in the highest excretion of lignans in rats, followed in decreasing order by oat, barley and wheat meal (19,55). However, the exact composition of the different meal products fed to the rats is not known.

Based on an epidemiological study it was recently suggested that consumption of fermented milk products may protect against BC (49,65). One mechanism by which fermented milk may influence hormone metabolism is by reduction of the β -glucuronidase-producing bacteria of the intestinal content (28,29), which should reduce the enterohepatic circulation of estrogens and increase the fecal route of elimination (14). However, milk products have also been found to contain animal lignans and isoflavonic phytoestrogens (5) and even if the concentrations are rather low they add to those produced by the intestinal bacteria from plant precursors.

Our hypothesis has been that high intake of whole-grain products (preferably in combination with reduced fat and moderate protein intake) reduces BC risk because such a diet increases fecal bulk and reduces intestinal β -glucuronidase activity and steroid enterohepatic circulation and results in increased mammalian lignan production (1,4). Later on we also included the isoflavonic phytoestrogens into the original theory (2,12). This was due to the finding of very high excretion of isoflavonic phytoestrogens in urine of Japanese men and women consuming a traditional diet (9,12, unpublished data). The lignan excretion in the Japanese subjects was low, even lower than we found in the postmenopausal BC patients in Boston. The isoflavones resemble lignans with regard to structure (all are diphenolic). In most correlation studies they show parallel behaviour. In the Finnish women the significances of the positive correlation between the excretion of lignans and isoflavonic phytoestrogens in urine, and plasma SHBG, and the negative correlations with %FE2 and %FT are stronger than the separate correlations for each group of compounds (12). Recently, our hypothesis with regard to the protective role of

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Dietary Estrogens: a Biologically Active Background for Estrogen Action¹

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INTRODUCTION

The discovery of phytoestrogens and other estrogenically active compounds in human urine has heightened concern about their potential impact on human reproductive function (3,63). Important issues that need to be addressed are the impact of these substances on estrogen action *in vivo* and the efficacy of natural concentrations of dietary estrogens. These substances could play a beneficial role by antagonizing the action of carcinogens (2,63) but might also play a harmful role by disrupting reproduction (62), stimulating cancer cell growth (62,71) or altering sexual differentiation (72). Even more fundamental is the question of whether natural concentrations of these substances are sufficient to exert any of these effects. Answers to the former question have been complicated by the discovery of other diphenolic plant chemicals like the lignans and bioflavonoids that antagonize estrogen action (2,36,48). Answers to the latter question have been impeded by the limited information available on the levels of these substances in human foods and the difficulty of relating data obtained by parenteral administration to the effects of natural dietary levels of phytoestrogens. We have attempted to address these questions with a comprehensive study of the effects of dietary estrogens on a variety of reproductive processes and reproductive and biochemical events. These studies reveal that dietary estrogens provide a widespread and biologically active background for estrogen action that impacts a wide range of physiologic functions.

ESTROGENIC SUBSTANCES IN HUMAN DIETS

A phenolic ring enables many environmental substances to bind to the estrogen receptor, and some are biologically active estrogen agonists or antagonists (49). We have termed these environmental compounds "xenoestrogens" to denote their

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15 β -Hydroxysteroids (Part III).^{*} Steroids of the human perinatal period: The synthesis of 3 β , 15 β , 17 α -trihydroxy-5-pregnen-20-one. Application of *n*-butyl boronic acid protection of a 17,20-glycol

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We report the synthesis of 3 β ,15 β ,17 α -trihydroxy-5-pregnen-20-one (1) from 3 β ,15 β -dihydroxy-5,16-pregnadien-20-one (11) in 7 steps using boronate derivatives as a means of protecting the 17,20-glycol side-chain of steroid intermediates. 16 α ,17 α -Epoxy-3 β ,15 β -dihydroxy-5-pregnen-20-one (12), an intermediate in the synthesis was prepared by epoxidation of 11 using a mixture of sodium hydroxide and hydrogen peroxide. Reduction of 12 with lithium aluminium hydride gave the two isomers of 5-pregnene-3 β ,15 β ,17 α ,20(S+R)-tetrol (13a and 13b) which on subsequent reaction with *n*-butyl boronic acid gave 5-pregnene-3 β ,15 β ,17 α ,20(S+R)-tetrol 17 α ,20-butyl boronate (15a and 15b). Acetylation with acetic anhydride and pyridine yielded 3 β ,15 β -diacetoxy-5-pregnene-17 α ,20(S+R)-diol 17 α ,20(S+R)-butyl boronate (15c and 15d). Oxidative cleavage of the boronic ester using sodium hydroxide and hydrogen peroxide gave 3 β ,15 β -diacetoxy-5-pregnene-17 α ,20(S+R)-diol (13c and 13d). After isolation of these latter two products, dibromide protection of the C-5,6 olefin of 13d and oxidation with *N*-bromosuccinimide gave 3 β ,15 β -diacetoxy-17 α -hydroxy-5-pregnen-20-one (16) which on deacetylation gave in good yield (35%) the desired product 3 β ,15 β ,17 α -trihydroxy-5-pregnen-20-one (1) in an overall yield of 24% from 11. (Steroids 61:11–17, 1996)

Keywords: adrenal, *n*-butyl boronate; 15 β -hydroxysteroids; hyperplasia; neonate; synthesis

Introduction

An increasing number of 15 β -hydroxylated steroids have been identified in human urine.^{1,2} Of these, 3 β ,15 β ,17 α -trihydroxy-5-pregnen-20-one (1) and 3 α ,15 β ,17 α -trihydroxy-5 β -pregnan-20-one (2) have emerged as the two

most important urinary metabolites. The former is a normal metabolite of the human perinatal period and a probable precursor of all known 15 β -hydroxylated steroids, while the latter is a metabolite pathognomonic of congenital adrenal hyperplasia (CAH) in newborn infants.¹ Other similar steroids identified more recently are those of 3 α ,15 β ,17 α -trihydroxy-5 α -pregnan-20-one (3), 3 β ,15 β ,17 α -trihydroxy-5 α -pregnan-20-one (4), 3 β ,15 β ,17 α -trihydroxy-5 β -pregnan-20-one (5), 5 α -pregnane-3 α ,15 β ,17 α ,20S-tetrol (6), and 5 β -pregnane-3 α ,15 β ,17 α ,20S-tetrol (7), found in the urine of a four-month-old girl affected with CAH due to 21-hydroxylase deficiency (Figure 1).²

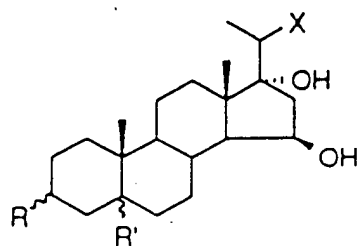
In our investigation of 15 β -hydroxylated steroids, the synthesis of 1 became the primary aim. To this end, the synthesis of 3 β ,15 β -dihydroxy-5,16-pregnadien-20-one

^{*}Parts I–VI in press, *Steroids*.

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	R	R'	X
1	3 β -OH	Δ^5	O
2	3 α -OH	5 β -H	O
3	3 α -OH	5 α -H	O
4	3 β -OH	5 α -H	O
5	3 β -OH	5 β -H	O
6	3 α -OH	5 α -H	(20S) OH,H
7	3 α -OH	5 β -H	(20S) OH,H
14	O	Δ^4	O

Figure 1 The chemical structures of compounds 1–7 and 14.

(11) from 3 β -hydroxy-5,15-androstadien-17-one (8), was achieved as a first-step objective (data submitted for publication) in a scheme where selective oxidation of isomeric 5-pregnen-3 β ,15 β ,17 α ,20(*S+R*)-tetrols (13a and 13b) with *N*-bromosuccinimide would yield the desired product (Scheme 1).³ In brief, reaction of 8 with 2-lithio-2-methyl-1,3-dithiane gave 20,20-trimethylenedithio-5,15-pregnadien-3 β ,17 β -diol (9) which when treated with aqueous acid rearranged to give 20,20-trimethylenedithio-5,16-pregnadien-3 β ,15 β -diol (10). Cleavage of the dithioacetal group with mercuric chloride gave 11 which on epoxidation using basic hydrogen peroxide afforded 16 α ,17 α -epoxy-3 β ,15 β -dihydroxy-5-pregnen-20-one (12). Reduction with lithium aluminium hydride gave the desired isomeric 5-pregnene-3 β ,15 β ,17 α ,20(*S+R*)-tetrols (13a and 13b). However, oxidation with *N*-bromosuccinimide, after protection of the C-5,6 olefin as the dibromide, gave 15 β ,17 α -dihydroxy-4-pregnen-3,20-dione (14) in only fair yield³ with no evidence of the desired product 1.

In this investigation we report the successful conversion of the isomeric tetrols (13a and 13b) to 1. This required the development of a protocol whereby the 3 β - and 15 β -hydroxy groups were protected prior to the oxidation of the 17 α ,20-glycol.

Experimental

The starting material, 8, was obtained in our laboratory as an intermediary product of synthesis of 14. The gas chromatography (GC), GC-mass spectroscopy (MS), elemental analysis, and NMR spectra of 14 were reported elsewhere.

Solvents were laboratory grade or better. Melting points were

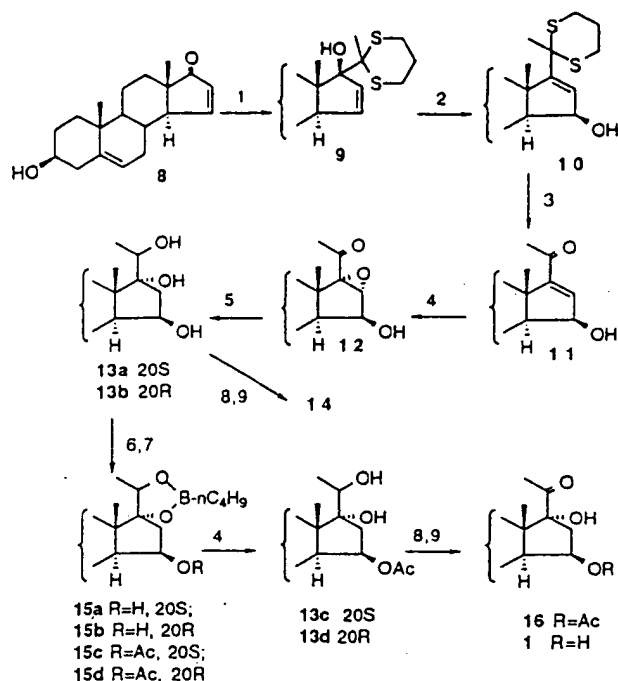
determined on a Gallenkamp Melting Point Apparatus and are uncorrected. Ultraviolet spectra were determined on a Varian Techtron ultraviolet-visible spectrophotometer. ¹H NMR were recorded at 200 MHz using a Bruker AC-200F spectrometer using TMS as internal reference and deuteriochloroform as the solvent unless otherwise noted. *W*_{1/2} refers to the peak width at its half height. Gas chromatography was performed on a Hewlett Packard 5710A flame ionization gas chromatograph using a glass solid injector and interpreted using a Shimadzu CR-4A chromatopac utilizing EurekaSoft analytical software (EurekaAnalytical, Camperdown, Australia), results are expressed as methylene units (MU).⁵ A 30 m capillary column from Heliflex Capillaries (RSL-150 polydimethylsiloxane; ID 0.25 mm) was used with helium as the carrier gas (2.0 mL/min), temperature programming from 197–270°C at 1°C/min with the detector and injector block temperatures were 300°C and 250°C, respectively. Mass spectra were recorded on a Finnigan MAT TSQ-70 mass spectrometer scanning from 80 to 800 *d* at 70 eV. CIMS was obtained using methane (CICH₄) or methane/ammonia (CINH₃) as plasma and with a reagent gas pressure of 5–10 torr. Low resolution CIMS using isobutane and high resolution mass spectra were run on a VG Autospec. Silica gel H (Merck, type 60) was used for chromatography.⁶ Steroid derivatization for GC and GC-MS analyses were either as the methyloxime-pertrimethylsilyl derivative (MOTMS) or as the pertrimethylsilylated derivative (TMS) and were carried out as reported earlier.¹

16 α ,17 α -Epoxy-3 β ,15 β -dihydroxy-5-pregnen-20-one (12)

A solution of 11 (198 mg, 0.6 mmol) in methanol (13 mL) was cooled in an ice bath then aqueous sodium hydroxide (600 μ L, 2 M) and aqueous hydrogen peroxide (500 μ L, 30%) was added. The reaction mixture stood for 16 h at 4°C. After neutralization with acetic acid (70%) and evaporation to dryness, the residue was partitioned between ethyl acetate and water. The organic phase was washed with water, dried (Na₂SO₄), and evaporated to dryness. Recrystallization using dichloromethane–light petroleum gave 160 mg (77%) of 12: m.p. 172–174°C; ¹H NMR δ 5.34 (1 H, *W*_{1/2} = 10 Hz, H-6), 4.20 (1 H, *d J* = 5 Hz, H-15), 3.71 (1 H, br s, H-16), 3.50 (1 H, *W*_{1/2} = 26 Hz, H-3), 2.05 (3 H, s, H-21), 1.27 (3 H, s, H-18), 1.05 (3 H, s, H-19); SP-EIMS *m/z* (%) 346 (37) [M⁺], 328 (26) [M-H₂O], 313 (11) [M-H₂O-Me], 310 (10) [M-2 \times H₂O], 285 (10) [M-H₂O-MeCO], 267 (20), 253 (17), 220 (21), 205 (76), 175 (27), 171 (25), 169 (14), 161 (15), 159 (22), 157 (14), 148 (44), 147 (33), 145 (39), 143 (27), 133 (20), 127 (20), 119 (43), 91 (100). Found C 72.5%, H 8.4%; C₂₁H₃₀O₄ requires C 72.8%, H 8.7%. GC (as MOTMS) MU = 29.70; GC-EIMS *m/z* (%) 519 (10) [M⁺], 504 (12) [M-Me], 488 (55) [M-OMe], 398 (20) [M-OMe-OTMS], 356 (20), 324 (16), 308 (23), 282 (17), 254 (26), 197 (19), 171 (58), 129 (100).

5-Pregnene-3 β ,15 β ,17 α -20(*S+R*)-tetrol (13a and 13b)

To a solution of 12 (250 mg, 0.72 mmol) in anhydrous tetrahydrofuran (50 mL), lithium aluminium hydride (30 mg, 0.8 mmol) was added and the mixture was refluxed for 1 h. The excess reagent was destroyed by the addition of ethyl acetate and the mixture filtered through celite, dried (Na₂SO₄), and evaporated to dryness. Chromatography of the residue on silica, eluting with ethyl acetate/light petroleum (3:1, v/v) gave after recrystallization from acetone/hexane 180 mg (71%) of 13b: m.p. 212–214°C; ¹H NMR δ 5.36 (1 H, *W*_{1/2} = 10 Hz, H-6), 4.28 (1 H, *W*_{1/2} = 12 Hz, H-15), 4.11 (1 H, *q J* = 6 Hz, H-20), 3.50 (1 H, *W*_{1/2} = 26 Hz, H-3), 1.16 (3 H, *d J* = 6 Hz, H-21), 1.06 (3 H, s, H-18), 1.05 (3 H, s, H-19); SP-EIMS *m/z* (%) 350 (6) [M⁺], 332 (7) [M-H₂O], 305



1) 2-methyl-1,3-dithiane, LDA, -78°C; 2) H⁺, H₂O; 3) HgCO₂, CaCO₃; 4) NaOH, H₂O₂; 5) LiAlH₄, THF; 6) n-C₄H₉B(OH)₂; 7) Ac₂O, Pyr; 8) Pyridine.HBr.Br₂, NBS, NaI; 9) NaOH

Scheme 1 The synthesis of 5-pregnen-3 β ,15 β ,17 α ,20(R+S)-tetrol (13a and 13b) and its conversion to 3 β ,15 β ,17 α -trihydroxy-5-pregnene-20-one (1) and 15 β ,17 α -dihydroxy-4-pregnen-3,20-dione (14).

(100) [M-CH₃CHOH], 287 (25) [M-CH₃CHOH-H₂O], 271 (16), 269 (20), 251 (11), 215 (20), 211 (16), 200 (15); SP-CIMS/CH₄ m/z (%) 349 (12) [M-1]⁺, 333 (92) [MH-H₂O], 315 (100) [M-CH₃CHOH], 287 (37), 269 (13); Found: C 72.3%, H 9.8%; C₂₁H₃₄O₄ requires C 72.0% H 9.8%. GC (as TMS) MU = 29.71; GC-EIMS m/z (%) 638 (1) [M]⁺, 521 (100) [M-CH₃C-OTMS], 431 (72) [M-117-OTMS], 391 (22), 333 (23), 251 (13), 191 (40), 147 (35), 117 (78).

Further elution gave after recrystallization from acetone/hexane 40 mg (17%) of 13a: m.p. = 223–224°C; ¹H NMR (CDCl₃) δ p.p.m. 5.36 (1 H, m W_{1/2} = 10 Hz, H-5), 4.28 (1 H, m W_{1/2} = 15 Hz, H-15), 3.91 (1 H, q J = 6 Hz, H-20), 3.50 (1 H, m W_{1/2} = 26 Hz, H-3), 1.19 (3 H, d J = 6 Hz, H-21), 1.05 (3 H, s, H-19), 0.99 (3 H, s, H-18); SP-EIMS m/z (%) 350 (not detected, ND), 305 (100) [M-CH₃CHOH], 287 (6) [M-45-H₂O], 269 (5), 211 (16); SP-CIMS/CH₄ m/z (%) 349 (13) [M-1]⁺, 333 (100) [M-CH₃], 315 (83) [M-15-H₂O], 297 (14) [M-15-2 \times H₂O], 271 (13) [M-15-3 \times H₂O], 245 (3); Found: C 68.4%, H 9.9% C₂₁H₃₄O₄ · H₂O requires C 68.4%, H 9.8%. GC (as TMS) MU = 29.84; GC-EIMS m/z (%) 638 (1) [M]⁺, 521 (100) [M-CH₃CHOTMS], 431 (72) [M-117-OTMS], 391 (22), 333 (23), 251 (13), 191 (40), 147 (35), 117 (78).

5-Pregnen-3 β ,15 β ,17 α ,20S-tetrol 17 α ,20S-butyl boronate (15a)

To a solution of 13a (10 mg, 0.028 mmol) in ethyl acetate (5 mL), *n*-butylboronic acid (3 mg, 0.029 mmol) was added and after 5 min standing at room temperature, the solution was evaporated to dryness to give 11.9 mg of a mixture of 15a and 13a in a ratio of 74:26 (analysis by GC and GCMS): Compound (15a): ¹H NMR (CDCl₃)

δ p.p.m. 5.34 (1 H, W_{1/2} = 10 Hz, H-6), 4.3 (2 H, W_{1/2} = 11 Hz, H-15 + 20), 3.48 (1 H, W_{1/2} = 23 Hz, H-3), 1.26 (3 H, d J = 6 Hz, H-21), 1.05 (3 H, s, H-19), 0.99 (3 H, s, H-18); SP-CIMS/CH₄ m/z (%) 416 (8) [M]⁺, 398 (24) [M-H₂O], 383 (10) [M-H₂O-Me], 315 (8) [M-H₂O-Me-nBuB], 297 (100), 287 (24), 269 (30), 253 (12), 215 (27), 183 (15), 145 (38); high resolution MS (SP-CIMS/CH₄) [as M]⁺ expected 416.3098; found = 416.3094. GC (as TMS) MU = 31.03; GC-EIMS m/z (%) 560 (<0.5) [M]⁺, 545 (3) [M-Me], 472 (12) [M-Me-Me₃Si], 470 (36) [M-Me₃SiOH], 455 (6) [M-Me₃SiOH-Me], 431 (5), 380 (12) [M-2 \times Me₃SiOH], 365 (21), 353 (16), 297 (9), 278 (53), 263 (64), 251 (40), 238 (30), 224 (23), 209 (40), 184 (19), 171 (28), 157 (47), 145 (33), 129 (100), 118 (30), 105 (40); GC-CIMS/CH₄ m/z (%) 561 (25) [MH]⁺, 559 (39), [M-1], 545 (100) [M-Me], 471 (54) [M+1-Me₃SiOH], 459 (17), 381 (59) [M+1-2 \times Me₃SiOH], 369 (23), 297 (10), 279 (21), 255 (7), 129 (10), 91 (25).

5-Pregnen-3 β ,15 β ,17 α ,20R-tetrol 17 α ,20R-butyl boronate (15b)

To a solution of 13b (100 mg, 0.28 mmol) in ethyl acetate (27 mL), *n*-butyl boronic acid (29.6 mg, 0.29 mmol) was added and after 5 min standing at room temperature, the solution was evaporated to dryness to give 119 mg (102%) of 15b and 13b in a ratio of 97:3 (as analyzed by GC and GC-EIMS as the persilylated derivatives [M⁺560] and [M⁺638], respectively). 15b: ¹H NMR δ 5.3 (1 H, W_{1/2} = 10 Hz, H-6), 4.35 (2 H, W_{1/2} = 10 Hz, H-15 + 20), 3.47 (1 H, W_{1/2} = 12 Hz, H-3), 1.29 (3 H, d J = 7 Hz, H-21), 1.07 (3 H, s, H-19), 0.93 (3 H, s, H-18); SP-CIMS/CH₄ m/z (%) 416 (11) [M⁺], 398 (37) [M-H₂O], 383 (11) [M-H₂O-Me], 315 (54) [M-H₂O-Me-nBuB], 297 (100), 287 (29), 269 (61), 253 (33), 215 (14), 183 (17), 154 (16), 145 (13), 129 (21), 119 (11); high resolution MS expected (SP-CIMS/CH₄) [as M]⁺ 416.3098; found = 416.3099. GC (as TMS) MU = 30.70. GC-EIMS m/z (%) 560 (0.4) [M]⁺, 545 (3) [M-Me], 470 (36) [M-Me₃SiOH], 455 (7%) [M-Me₃SiOH-Me], 431 (7), 380 (17), 365 (29), 353 (18), 297 (8), 281 (11), 278 (35), 263 (64), 251 (33), 238 (20), 224 (25), 209 (46), 184 (19), 171 (28), 157 (44), 147 (38), 129 (100), 105 (40). GC-CIMS/CH₄ m/z (%) 561 (28) [MH]⁺, 559 (38), 545 (100), 470 (43), 459 (32), 381 (59), 369 (26), 297 (14), 279 (18).

3 β ,15 β -Diacetoxy-5-pregnen-17 α ,20(S)-diol 17 α ,20(S)-butyl boronate (15c)

A solution of the crude boronate 15a (15 mg, 0.036 mmol, 74% by GC) in pyridine (5 mL) and acetic anhydride (5 mL) stood for 48 h in the dark at room temperature. The mixture was then evaporated to dryness to give 18 mg of the 15c as a gum [GC/MS analysis shows 63% pure with two other compounds 17% of 15a and 20% of 3 β ,15 β ,20S-triacetoxy-5-pregnen-17 α -ol (13e)]: Compound (15c): ¹H NMR (CDCl₃) δ p.p.m. 5.40 (1 H, m W_{1/2} = 10 Hz, H-6), 5.15 (1 H, m W_{1/2} = 16 Hz, H-15), 4.61 (1 H, m W_{1/2} = 28 Hz, H-3), 4.32 (1 H, q J = 6.6 Hz, H-20), 2.04 (3 H, s, OAc), 2.01 (3 H, s, OAc), 1.32 (3 H, d J = 6.6 Hz, H-21), 1.04 (3 H, s, H-19), 0.88 (3 H, s, H-18); SP-CIMS/CH₄ m/z (%): 501 (3) [MH]⁺, 441 (53) [M-CH₃CO₂H], 399 (10) [M-C₄H₉B(OH)₂], 381 (100) [M-2 \times CH₃CO₂H], 339 (39), 313 (7), 297 (25), 279 (42); DCI probe-Cl/Isobutane [as MH] m/z (%) 501 (0.5) [MH]⁺, 441 (36) [M-CH₃CO₂H], 399 (19) [M-nBuBO₂H₂], 381 (100) [M-2 \times CH₃CO₂H], high resolution MS (DCI probe-Cl/Isobutane) expected 501.3387; found = 501.3382. GC (not derivatized) MU = 32.84; GC-EIMS m/z (%): 500 (ND) [M]⁺, 380 (69) [M-2 \times CH₃CO₂H]⁺, 365 (18), 343 (7), 279 (15), 263 (100), 251 (14), 236 (21), 220 (31), 209 (57), 193 (32), 170 (51), 155 (33), 145 (63), 129 (94), 105 (58).

**3 β , 15 β -Diacetoxy-5-pregnene-17 α , 20(R)-diol
17 α , 20(R)-butyl boronate (15d)**

To a solution of the crude boronate 15b (100 mg, 0.23 mmol, 97% by GC) in pyridine (5 mL), acetic anhydride (5 mL) was added and the mixture stood for 48 h in the dark at room temperature. The mixture was then evaporated to dryness to give 115 mg (95%) of 15d as a gum [GC/MS analysis shows 97% pure with 3% of 13f]. Compound (15d): $^1\text{H NMR}$ δ 5.40 (1 H, m, $W_{1/2}$ = 11 Hz, H-6), 5.24 (1 H, m, $W_{1/2}$ = 18 Hz, H-15), 4.65 (1 H, m, $W_{1/2}$ = 28 Hz, H-3), 4.40 (1 H, q, J = 6.6 Hz, H-20), 2.04 (3 H, s, OAc), 2.01 (3 H, s, OAc), 1.25 (3 H, d, J = 6.6 Hz, H-21), 1.07 (3 H, s, H-19), 0.87 (3 H, s, H-18); SP-CIMS/ CH_4 m/z (%) 500 (2) [M-1], 501 (ND) [M+1] $^+$, 441 (21) [M-CH₃CO₂H], 399 (3) [M-nBuBO₂H₂], 381 (100) [M-2 \times CH₃CO₂H], 339 (28) [M-nBuBO₂H₂-CH₃CO₂H], 297 (13), 279 (11); DCI probe-CI/isobutane m/z (%) 501 (0.5) [MH $^+$], 441 (36) [M-CH₃CO₂H], 399 (19) [M-nBuBO₂H₂], 381 (100) [M-2 \times CH₃CO₂H], high resolution MS (DCI probe-CI/isobutane) [as MH] expected 501.3387; found = 501.3379. GC (not derivatized) MU = 32.40; GC-EIMS m/z (%) 500 (ND) [M $^+$], 440 (2) [M-CH₃CO₂H] $^+$, 398 (5) [M-nBuBO₂H₂], 380 (100) [M-2 \times CH₃CO₂H], 365 (14), 278 (7), 263 (86), 251 (13), 209 (49), 183 (16), 155 (26), 129 (38); GC-CIMS/ CH_4 m/z (%) 500 (1), 441 (11), 383 (30), 381 (100), 339 (18), 297 (22), 279 (69); 253 (8), 106 (20).

3 β , 15 β -Diacetoxy-5-pregnene-17 α , 20(S)-diol (13c)

To a solution of the crude acetoxyboronate 15c (20 mg, 0.025 mmol, 63% by GC) in THF (3 mL), sodium hydroxide (2 M, 100 μL) was added followed by hydrogen peroxide (30%, v/v; 100 μL). After 1 h at room temperature the solution was neutralized with hydrochloric acid (1 M, 200 μL), evaporated to low volume and extracted with ethyl acetate. The organic phase was washed with water, dried (Na_2SO_4) and evaporated to dryness. The residue was chromatographed on silica gel, eluting with ethyl acetate/light petroleum (1:2, v/v) to give 7 mg (40%) of 13c: $^1\text{H NMR}$ δ 5.39 (1 H, $W_{1/2}$ = 10 Hz, H-6), 5.17 (1 H, $W_{1/2}$ = 16 Hz, H-15), 4.63 (1 H, $W_{1/2}$ = 28 Hz, H-3), 3.92 (1 H, q, J = 6 Hz, H-20), 2.04 (3 H, s, OAc), 2.00 (3 H, s, OAc), 1.21 (3 H, d, J = 6 Hz, H-21), 1.07 (3 H, s, H-19), 0.96 (3 H, s, H-18). (d_6 -acetone) δ p.p.m. 5.37 (1 H, $W_{1/2}$ = 9 Hz, H-6), 5.08 (1 H, $W_{1/2}$ = 16 Hz, H-15), 4.50 (1 H, $W_{1/2}$ = 32 Hz, H-3), 3.83 (1 H, q, J = 6 Hz, H-20), 1.96 (3 H, s, OAc), 1.95 (3 H, s, OAc), 1.14 (3 H, d, J = 6 Hz, H-21), 1.07 (3 H, s, H-19), 0.98 (3 H, s, H-18); SP-CIMS/isobutane m/z (%) 417 (10) [M-H₂O], 399 (6) [M-2H₂O], 375 (100) [M-CH₃CO₂H], 357 (36) [M-H₂O-CH₃CO₂H], 339 (10) [M-2 \times H₂O-CH₃CO₂H], 315 (59) [M-2 \times CH₃CO₂H], 297 (61) [M-H₂O-2 \times CH₃CO₂H], 279 (8), 253 (15); SP-CIMS/ NH_3 m/z (%) 452 (100) [MNH₄ $^+$], 392 (18), 357 (4), 279 (7); high resolution MS (SP-CIMS/isobutane) [as MH-18, no MH present] expected = 417.2641; found = 417.2646. GC (as TMS): MU = 31.52; GC-EIMS m/z (%) 578 (ND) [M $^+$], 518 (0.4) [M-60] $^+$, 401 (87), 368 (9), 341 (26), 268 (5), 251 (17), 209 (4), 195 (15), 169 (27), 157 (27), 147 (39), 117 (100).

3 β , 15 β -Diacetoxy-5-pregnene-17 α , 20(R)-diol (13d)

To a solution of the crude diacetoxyboronate 15d (100 mg, 0.2 mmol, 97% by GC) in tetrahydrofuran (10 mL), sodium hydroxide (2 M, 0.5 mL) was added followed by hydrogen peroxide (30%, v/v; 0.5 mL). After 1 h at room temperature the solution was neutralized with hydrochloric acid (1 M, 1 mL), evaporated to low volume and extracted with ethyl acetate (3 \times). The organic phase was washed with water, dried (Na_2SO_4) and evaporated to dryness. The residue was chromatographed on silica gel, eluting with ethyl acetate/light petroleum (1:2, v/v) to give 60 mg (70%) of 13d: $^1\text{H NMR}$ δ 5.36 (1 H, $W_{1/2}$ = 9 Hz, H-6), 5.12 (1 H, $W_{1/2}$ =

15 Hz, H-15), 4.6 (1 H, $W_{1/2}$ = 28 Hz, H-3), 4.1 (1 H, q, J = 6 Hz, H-20), 2.03 (3 H, s, OAc), 1.99 (3 H, s, OAc), 1.16 (3 H, d, J = 6 Hz, H-21), 1.07 (3 H, s, H-19), 1.02 (3 H, s, H-18). (d_6 -acetone) 5.34 (1 H, $W_{1/2}$ = 8 Hz, H-6), 5.06 (1 H, $W_{1/2}$ = 12 Hz, H-15), 4.50 (1 H, $W_{1/2}$ = 32 Hz, H-3), 4.02 (1 H, q, J = 5 Hz, H-20), 1.96 (3 H, s, OAc), 1.94 (3 H, s, OAc), 1.09 (3 H, d, J = 5 Hz, H-21), 1.07 (3 H, s, H-19), 1.05 (3 H, s, H-18); SP-CIMS/isobutane m/z (%): 417 (10) [M-H₂O], 399 (6) [M-2H₂O], 375 (100) [M-CH₃CO₂H], 357 (36) [M-H₂O-CH₃CO₂H], 339 (10) [M-2 \times H₂O-CH₃CO₂H], 315 (59) [M-2 \times CH₃CO₂H], 297 (61) [M-H₂O-2 \times CH₃CO₂H], 279 (8), 253 (15); SP-CIMS/ NH_3 m/z (%): 452 (100) [MNH₄ $^+$], 392 (12), 314 (3); high resolution MS (SP-CIMS/isobutane) [as MH-18, no MH present] expected = 417.2641; found = 417.2634. GC (as TMS) MU = 31.49; GC-EIMS m/z (%): 578 (ND) [M $^+$], 518 (2) [M-60] $^+$, 502 (1), 457 (2.5), 401 (100), 368 (7), 341 (20), 195 (3), 169 (6), 157 (7), 131 (10), 117 (60).

**3 β , 15 β -Diacetoxy-5-pregnene-17 α , 20(S)-diol
17 α , 20S-n-butyl boronate (15c)**

A solution of 13c (2 mg, 0.0046 mmol) was dissolved in ethyl acetate (1 mL) and *n*-butyl boronic acid (0.6 mg) was added. After 5 min at room temperature GC analysis proved the mixture to consist of 13c and 15c in a ratio of 1:2.

3 β , 15 β -Diacetoxy-17 α -hydroxy-5-pregnen-20-one (16)

To a solution of 13d (50 mg, 0.115 mmol) in methanol (5 mL, 0.115 mmol), pyridinium bromide perbromide (50 mg, 0.23 mmol) was added. After 10 min at room temperature, the solution was evaporated to dryness, extracted with dichloromethane, and washed with water. The organic layer was evaporated to dryness and the residue was dissolved in dioxan (1 mL), water (100 μL) was added, followed by *N*-bromosuccinimide (35 mg, 0.2 mmol). The solution was stirred in the dark for 24 h before being diluted with ethyl acetate. Solid sodium iodide (20 mg, 0.13 mmol) was added and the solution was stirred for 5 min then aqueous saturated sodium thiosulfate was added. The reaction mixture was stirred for a further 5 min before it was extracted three times with ethyl acetate. The combined organic phase was washed with sodium bicarbonate (1.2 M) then water, dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed on silica gel eluting with dichloromethane/ethyl acetate to give after recrystallizing from dichloromethane/hexane 30 mg (60%) of 16: m.p. 277–278°C; $^1\text{H NMR}$ δ 5.38 (1 H, $W_{1/2}$ = 9 Hz, H-6), 5.23 (1 H, $W_{1/2}$ = 17 Hz, H-15), 4.61 (1 H, $W_{1/2}$ = 34 Hz, H-3), 2.28 (3 H, s, H-21), 2.04 (6 H, s, OAc-3,15), 1.07 (3 H, s, H-19), 0.92 (3 H, s, H-18); SP-CIMS/ CH_4 m/z (%): 433 (5) [MH $^+$], 415 (5) [M-H₂O], 373 (65) [M-CH₃CO₂H], 355 (28) [M-H₂O-CH₃CO₂H], 313 (100) [M-2 \times CH₃CO₂H], 295 (65); calculated for C₂₅H₃₆O₆: C, 69.42; H, 8.39. Found: C, 69.72; H, 8.33. GC (as the methyloxime) MU = 31.05; GC-CIMS/ CH_4 m/z (%): 533 (30) [MH $^+$], 502 (7), 473 (13) [M-CH₃CO₂H], 442 (100) [M-(CH₃)₃SiOH], 383 (9), 352 (34), 323 (18), 309 (12), 292 (55), 251 (10), 212 (11), 187 (29), 186 (38), 170 (49), 169 (34), 157 (26), 145 (35), 131 (21), 121 (23), 105 (24).

3 β , 15 β , 17 α -Trihydroxy-5-pregnen-20-one (1)

To a solution of 16 (30 mg, 0.07 mmol) in ethanol (4 mL), sodium hydroxide (0.5 mL, 2 M) was added and the solution was heated at 40°C for 10 min. Hydrochloric acid (1 M, 1 mL) was added, the mixture was evaporated to low volume and diluted with ethyl acetate. The organic phase was washed with water, dried (Na_2SO_4), and evaporated to dryness to give after crystallization from acetone 22 mg (91%) of 1: m.p. 258–260°C; $^1\text{H NMR}$ δ 5.37 (1 H, m, $W_{1/2}$ = 10 Hz, H-6), 4.36 (1 H, m, $W_{1/2}$ = 14 Hz, H-15), 3.50

(1 H, m $W_{1/2}$ = 24 Hz, H-3), 2.28 (3 H, s, H-21), 1.04 (3 H, s, H-19), 0.94 (3 H, s, H-18); SP-CIMS/ CH_4 m/z (%): 349 (6) $[\text{M}+1]^+$, 347 (21), 332 (37), 331 (100), $[\text{M}-\text{H}_2\text{O}]$, 313 (93) $[\text{M}-2 \times \text{H}_2\text{O}]$, 295 (57) $[\text{M}-3 \times \text{H}_2\text{O}]$, 271 (9), 253 (5). SP-CIMS/ NH_3 m/z (%): 366 (100) $[\text{MNH}_4]^+$, 348 (39), 331 (49), 313 (10), 295 (3); high resolution EIMS expected 348.2300; found = 348.231 GC (as MOTMS) MU = 29.11; GC-EIMS m/z (%): 593 (26) $[\text{M}^+]$, 562 (76), 503 (10), 472 (38), 362 (19), 258 (100), 231 (12), 188 (31), 129 (29). The GC and GC/MS data are identical to that reported earlier.¹

Results and discussion

The synthesis of 3 β ,15 β ,17 α -trihydroxy-5-pregnen-20-one (1) from 5-pregnene-3 β ,15 β ,17 α ,20(*S*+*R*)-tetrols (13a and 13b) required the selective protection of the C-3 β and C-15 β hydroxy groups prior to the oxidation of the C-20 hydroxy functional group. In principle, this could be achieved by either the direct preparation of protected derivatives of 13a and 13b or by preparation of suitable derivatives of 3 β ,15 β -dihydroxy-5,16-pregnadien-20-one (11) or 16 α ,17 α -epoxy-3 β ,15 β -dihydroxy-5-pregnen-20-one (12) followed by reduction to the protected 13a and 13b. Attempts to prepare derivatives of 11 and 12 followed by reduction in good yield included the formation and cleavage of the *t*-butyldimethylsilyl, tetrahydropyranyl, methoxymethylene ethers, and acetyl esters but were unsuccessful.

Since a high-yielding synthesis of protected forms of the C-3 and C-15 hydroxy was not found, the protection of the C-17,20 glycol was investigated prior to reaction of the C-3 and C-15 alcohols. The preparation of acetonide ketals was not successful due to the ease of dehydration of the C-15 β alcohols. A scheme was devised using boronate derivatives to protect the glycol side chain prior to protection of the C-3 and C-15 hydroxy functional groups which after hydrolysis of the boronate ether would yield suitable derivatives of 13a and 13b. To this end a number of boronate derivatives were investigated.

Both *n*-alkylboronates and *n*-aryl boronates are known for the protection of diols or amino alcohols.⁷⁻¹⁷ There are two methods employed to prepare the boronates. An ethyl acetate solution of the diol or amino alcohol is stirred with an *n*-alkyl boronic acid or *n*-aryl boronic acid^{9,14,16} or the diol or amino alcohol is refluxed in the presence of the corresponding trialkyl boroxine, the anhydride of the corresponding boronic acid.^{7,11,12} However, a side-product of this reaction is *n*-butyl boronic acid and, although only a weak acid, under these reaction conditions it was expected that catalytic dehydration of the C-15 β hydroxy group could occur.

The quantitative conversion of 5 β -pregnane-3 α ,17 α ,20(*S*+*R*)-triol (18a and 18b) with *n*-butyl boronic acid to the C-20*R* and C-20*S* isomers of 5 β -pregnane-3 α ,17 α ,20(*S*+*R*)-triol-17 α ,20-butyl boronate 19a and 19b has been reported previously.¹⁶ It was shown that the free alcohols of these compounds were easily converted to the trimethylsilyl ether or acetate derivatives. It is known that the benzene boronic esters tend to be resistant to hydrolysis when compared to the butyl boronic ester¹⁴; however, as the planned reaction sequence required the selective hydrolysis of the boronic ester in preference to the C-3 β and C-15 β acetates using a mixture of sodium hydroxide and hydrogen perox-

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ide, the choice of boronic acid (e.g., methyl, *n*-butyl, *t*-butyl, or benzene boronic acids) was based upon the ease of formation and removal versus its stability to hydrolysis.

In this study, reaction of *n*-butyl boronic acid with 5-pregnene-3 β ,15 β ,17 α ,20*R*-tetrol (13b) resulted in a product which on analysis by GC and GC-MS, after reaction with bis-(trimethylsilyl)-trifluoroacetamide, proved to be a mixture of 97% 5-pregnene-3 β ,15 β ,17 α ,20*R*-tetrol-17 α ,20*R*-butyl boronate (15b) and 3% 13b. Acetylation of this mixture with acetic anhydride in pyridine gave 3 β ,15 β -diacetox-5-pregnene-17 α ,20*R*-diol 17 α ,20*R*-butyl boronate (15d) with 3 β ,15 β ,20*R*-triacetox-5-pregnene-17 α -ol (13f) as a minor component (3%). The boronate (15d) was rapidly cleaved on treatment with sodium hydroxide and hydrogen peroxide to give after chromatographic purification 3 β ,15 β -diacetox-5-pregnene-17 α ,20*R*-diol (13d) in 70% overall yield (Scheme 1).

In contrast to the reactions observed with 13b, reaction of the 20*S*-tetrol (13a) under identical conditions with *n*-butyl boronic acid in ethyl acetate gave a mixture of 74% of 5-pregnene-3 β ,15 β ,17 α ,20*S*-tetrol 17 α ,20*S*-butyl boronate (15a) and 26% of 13a. Acetylation using acetic anhydride in pyridine could not be induced to go to completion and resulted in a mixture of 3 β ,15 β -diacetox-5-pregnene-17 α ,20*S*-diol-17 α ,20*S*-butyl boronate (15c), 15a and 3 β ,15 β ,20*S*-triacetox-5-pregnene-17 α -ol (13e) as identified by GC-MS. Hydrolysis of this mixture with sodium hydroxide and hydrogen peroxide gave after chromatography 3 β ,15 β -diacetox-5-pregnene-17 α ,20*S*-diol (13c) in 40% overall yield.

The above results are for reactions of 13a and 13b with *n*-butyl boronic acid run at the same time and identical conditions; therefore, these results reflect the equilibrium position of these reactions. Numerous repetitions of these reactions under different conditions, i.e., changing reagent concentrations and so on gave similar results. Under identical reaction conditions to that used in the reactions of 13a and 13b, we were able to reproduce the reported results¹⁶ that 18a and 18b react quantitatively with *n*-butyl boronic acid to give 19a and 19b. The rate of hydrolysis of the acetonides of 18a and 18b have been found to be vastly different. The acetonide of 18a was hydrolyzed using 60–80% aqueous acetic acid at room temperature within 24 h. In stark contrast, the acetonide of 18b was stable under these conditions, requiring refluxing 80% acetic acid to hydrolyze. These results were interpreted in terms of the ease of approach of the proton to the 20*S* acetonide and also due to the additional ring strain of the acetonide due to the steric interaction of the C-18 and C-21 methyl groups.²⁰

Inspection of molecular models reveals that the 17 α ,20*S*-glycol is locked in a *cis* conformation and there exists a serious non-bonded interaction between the C-18 and the C-21 methyl groups (Figure 2). Addition of a 15 β -hydroxy group as in 15a adds another 1-3 diaxial non-bonded interaction between the C-15 β hydroxy group and the C-18 methyl group, increasing the steric congestion of the C-18 methyl group, which results in destabilization of the C-17,20*S*-boronate. The difference in ease of acetylation of 15a and 15b is indicative of an increase in steric congestion of the C-15 β -hydroxy group by the C-18 methyl group in 15a as compared to 15b, where the C-18/C-21 intense meth-

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yl/methyl interaction is replaced with a minor methyl/hydrogen interaction. Further evidence supporting this premise was obtained by reaction of 13c, a compound in which the C-15 β hydroxy group has been acetylated to the C-15 β acetate, thereby slightly increasing the 1,3-diaxial interaction of the C-15 β group with the C-18 methyl groups, under identical conditions to those reactions reported above. The equilibrium position, as reflected by the ratio of products obtained 13c (33%) and 15c (67%), has been significantly shifted towards the unreacted diol. The relative stability of these molecules is complicated by the combination of a spiroannular bicyclopentane unit in which one of the cyclopentanes is part of a strained trans-8-methylhydrindane unit. An in-depth investigation using molecular dynamics would be needed to gain greater insight of the factors affecting the equilibria.

Although benzene boronic acid is known to yield esters of greater stability than the *n*-butyl boronic esters, reaction of 13a with benzene boronic acid in ethyl acetate yielded 5-pregnene-3 β ,15 β ,17 α ,20(*S*)-tetrol 17 α ,20*S*-benzene boronate and the starting material 13a in a similar ratio to that obtained with *n*-butyl boronic acid with no added advantage and therefore were not exploited in our studies.

N-Bromosuccinimide has been shown to be effective in glycol oxidation while at the same time avoiding glycol cleavage.^{18,19} Neither chromium compounds nor silver carbonate were successful in avoiding the cleavage of the glycol.³ Early attempts using *N*-bromosuccinimide gave numerous side products and low yields (10%); however, protection of the C-5 alkene as the dibromide resulted in a much cleaner reaction.³ In this study the reaction of 13d with pyridinium hydrobromide perbromide gave 3 β ,15 β -diacetox-5,6-dibromopregnane-17 α ,20*R*-diol which without isolation was oxidized with *N*-bromosuccinimide to give after debromination with sodium iodide, 3 β ,15 β -diacetox-17 α -hydroxy-5-pregnen-20-one (16) in 60% yield (Scheme 1). Hydrolysis of 16 with sodium hydroxide in ethanol gave 3 β ,15 β ,17 α -trihydroxy-5-pregnen-20-one (1), the desired

product, in an overall yield of (35%) from 13b. No attempts were made to obtain the same desired product from the corresponding isomer 13a.

This study shows that the application of boronate chemistry as a means of protecting the 17,20-glycol has been successful in assisting with the synthesis of 15 β -hydroxylated C-21 steroids. This overcame the major problem of protecting the 15 β -hydroxy group which proved difficult to derivatize due to steric hindrance, a rate-limiting step with other competing functional groups such as those at C-3 and C-20, respectively. It is envisaged that this method can also be applied in the synthesis of isomeric C-21 steroids hydroxylated at C-15 α .

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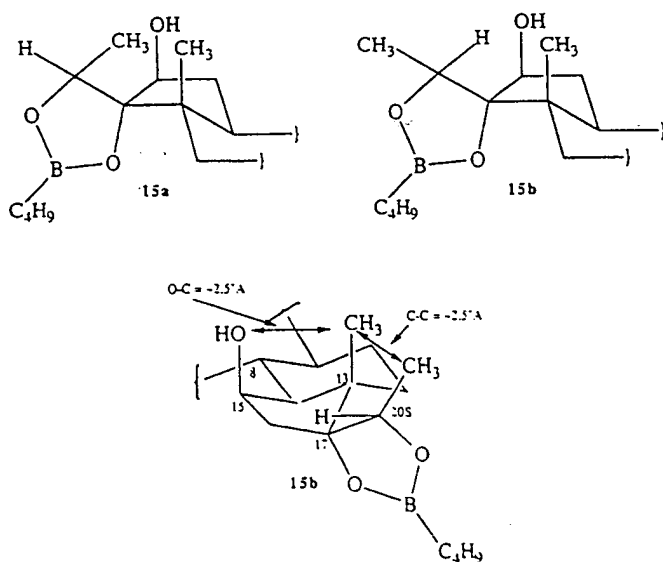


Figure 2 The stereochemical relationship of the C-21, C-18 methyls and the C-15 β hydroxy groups.

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Metabolites of dietary (soya) isoflavones in human urine

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Abstract

This study was undertaken to better understand the metabolic fate of dietary isoflavones in humans. Twelve volunteers were challenged with soya flour and urinary diphenol levels were then determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The presence of previously described urinary diphenols was confirmed, i.e. the isoflavones, daidzein and genistein; the isoflavonoid metabolites, equol, dihydrodaidzein (Int-O-D), *O*-desmethyl-angolensin (O-Dma); the lignan, enterolactone. Diphenols detected for the first time were the isoflavone, glycitein and five novel isoflavonoid metabolites which are tentatively identified as 6'-hydroxy-*O*-desmethylanlolensin (6'OH-O-Dma), dihydrogenistein (Int-O-G), dehydro-*O*-desmethylanlolensin (dehydro-O-Dma) and two isomers of tetrahydrodaidzein. Urinary excretion rates of the three isoflavones (daidzein, genistein, glycitein) over a 3-day period following soya challenge showed moderate variation (4×, 6× and 12×, respectively) between the 12 individuals suggesting some individual variabilities in ability to deconjugate and to absorb dietary isoflavones. However, urinary excretion rates of each of three major isoflavonoid metabolites (equol, O-Dma, 6'OH-O-Dma) showed more marked variation (922×, 17×, 15×, respectively); while some of this variability may reflect varying individual ability to ferment dietary isoflavones per se, an inverse relationship was found between urinary levels of equol and both O-Dma and 6'OH-O-Dma suggesting individual variability in the preferred metabolic pathways of dietary isoflavones.

Key words: Isoflavonoids; Daidzein; Genistein; Equol

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1. Introduction

Isoflavones are plant diphenols, with at least 230 types described [1]. A range of biological functions in plants has been ascribed to isoflavones, with anti-microbial activities being important [1]. A small number of isoflavones additionally display oestrogenic activity in animals, an effect which has been attributed to the similar spatial arrangement of functional groups on both isoflavones and oestrogens, allowing those isoflavones to bind to oestrogen receptors [2,3].

Isoflavones occur principally, although not exclusively, in legumes (*Leguminosae* family). Moreover, they are at particularly high levels in certain legumes which are regularly consumed by man and animals [4]. Indeed, many traditional human diets such as those in India, Asia, Africa, South and Central America and the Mediterranean, which have relatively high legume consumption (soya, lentils, chick peas, beans, etc.) consequently have high isoflavone contents, particularly those with oestrogenic activity [4].

There is growing interest in the importance of dietary isoflavones to human health. Rather than being just passive dietary components, isoflavones and in particular the oestrogenic isoflavones, have been hypothesised to have a range of beneficial physiological effects, largely related to their influence on sex hormone metabolism [5–9].

Interest in isoflavones as dietary components with active physiological effects stems from the 1950s with the finding that infertility in sheep grazing on subterranean clover was a direct result of high isoflavone levels in the pasture [10,11]. The principal isoflavones found in subterranean clover (*Trifolium*) are genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) which are present principally as glycoside conjugates (genistin, daidzin) or their respective 4'-methyl ethers, biochanin A and formononetin [11–14]. Upon ingestion the isoflavones are subjected to acidic hydrolysis and enzymatic hydrolysis and demethylation (from both plant and gut flora enzymes) to yield free aglycones and further metabolites [12–14]. Genistein is metabolised to the non-oestrogenic compound, *para*-ethylphenol, while daidzein is metabolised to the oestrogenic compounds, equol (4'',7-dihydroxyisoflavan) and *O*-desmethylangolensin [1-(2',4'-dihydroxyphenyl)-2-(4'-hydroxy-phenyl)propan-1-one] (*O*-Dma) [13,14]. The aglycones and their metabolites are absorbed and appear in blood and urine as glucuronide conjugates [12,13]. Those aglycones (genistein, daidzein) and metabolites (equol, *O*-Dma) with affinity for oestrogen receptors, then result in a range of pathophysiological effects in both ewes and wethers (including permanent infertility) as a result of the unresponsiveness of tissues such as the hypothalamus to endogenous oestrogen [15].

The metabolism of dietary isoflavones in humans is not as well understood as it is in sheep although the available evidence points to similar pathways in both species. Following ingestion of isoflavone-rich foods such as soya the isoflavones daidzein and genistein and the daidzein metabolites equol, *O*-Dma, as well as two intermediate compounds between daidzein and *O*-Dma (Intermediate *O*) and daidzein and equol (Intermediate *E*), have been described in human urine [16–20].

In view of the known *in vivo* potency of these isoflavones [15], the relatively high levels of these compounds in many traditional human diets suggests that a better understanding of isoflavonoid metabolism in humans is needed.

The purpose of the study reported here was to investigate both the scope and uniformity of the metabolic response in humans to dietary isoflavones.

2. Materials and methods

2.1. Experimental procedures

Participants

The participants were 12 healthy Caucasian men and women aged between 25 and 51 years of age, of normal height and weight. None of the participants was taking any medication including oral contraceptives; none had received antibiotic therapy within 6 months prior to the study. All participants were volunteers and provided informed consent. The study was approved by The University of Sydney Human Ethics Review Committee.

Diet

All participants had a typical Western European omnivorous diet, which included little or no soya products and only moderate legume intake estimated at 5–10 g (garden peas, French beans) daily. During the study, participants maintained their normal diet and had no more than moderate alcohol intake. Each participant included 40 g of soya flour in the form of a prepared cake in their daily diet for 2 consecutive days. The soya was a commercial brand of full-fat soya flour and the one batch of flour was used for all participants. The soya was analysed for isoflavone content as detailed later.

Urine sampling

Urine samples (24 h) were collected from each participant on four occasions. If the 2 consecutive days of soya challenge are designated as days 1 and 2, then the urine collections were on days 0, 3, 4 and 5. Sample sizes varied between 600 and 1,850 ml. The collection was made into 5-l polypropylene containers with no preservative added. The containers were kept cool and delivered to the laboratory on a daily basis. The volume of urine was recorded immediately and a 200-ml aliquot stored at –20°C until ready for analysis. The analysis of samples commenced within 3 months of collection.

2.2. Analytical methods

Reference standards and compound identity

Equol, daidzein and genistein were kindly supplied by Dr Lamberton, Division of Organic Chemistry, CSIRO, Australia. Dihydrodaidzein was a gift from Professor T. Hase, Department of Chemistry and Professor H. Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Finland. *O*-Desmethylangolensin and enterolactone were identified here by comparison of GC and GC-MS data from those in the literature [19,22–24].

Glycitein was extracted from soya and used as a reference standard after structural confirmation by NMR and GC-MS as follows. Isoflavones were extracted from soya

hypocotyl and hydrolysed by the method detailed later for the extraction and hydrolysis of daidzein and genistein from soya flour. GC and GC-MS showed that the extract consisted mainly of daidzein and glycitein and the glycitein was isolated by re-extraction with methanol followed by recrystallisation in aqueous (90%) methanol. The melting points and spectral analyses obtained were as follows: m.p. 290°C (dec.); UV λ_{\max} 256,319 (log ϵ 4.35, 3.98); diacetate m.p. 208–210°C; $^1\text{H-NMR}$ δ p.p.m. 8.02 (1H, s, H-2), 7.78 (1H, s, H-5), 7.62 (2H, d J = 8 Hz, H-2', 6'), 7.18 (2H, d J = 8 Hz, 3', 5'), 3.95 (3H, s, OCH_3), 2.37 (3H, s, OAc), 2.32 (3H, s, OAc). GC and GC-MS data were as follows. As the TMS ether derivative MU 30.37: M^+ 428 (100%), m/z 192 (85%), m/z 184 (43%), m/z 398 (35%), m/z 413 (22%) and m/z 383 (11%). As the diacetate derivative MU 30.20: M^+ 368 (13%), m/z 284 (100%), m/z 326 (28%), m/z 166 (26%), m/z 123 (9%), m/z 255 (7%), m/z 151 (5%), m/z 241 (4%) and m/z 118 (4%). The NMR and mass spectral data of glycitein diacetate obtained here are in agreement with data reported previously [24].

Isolation of diphenols from human urine

Enzyme hydrolysis and extraction. Aliquots of urine (40 ml) were adjusted to pH 4.6 with 70% acetic acid and 30,000 counts/min [^3H]oestradiol glucuronide (New England Nuclear) and 60 μg of oestriol added as internal standards. Enzymatic hydrolysis of the glucuronides was achieved using *Helix pomatia* juice (Calbiochem) in 0.1 M sodium acetate buffer, pH 5.0; 1000 Fishman units of beta-glucuronidase aryl sulphatase were added per ml of urine and incubated at 37°C for 24 h, after which time a fresh amount of enzyme was added and incubated for a further 24 h. Following extraction with diethyl ether (2 \times vol) and evaporation of the organic phase under nitrogen, the dry residue was dissolved in 1 ml of ethanol and stored at 5°C prior to partition chromatography.

Liquid-gel chromatography on Sephadex LH-20. The diphenol fraction, free of neutral steroids, was isolated by straight-phase partition chromatography on Sephadex LH-20. A siliconised Pasteur pipette (5 ml Corning) carrying a plug of glass wool at its narrow end served as a column, where the suspended slurry (1 g) was allowed to settle to 6.0 cm by gravity. A 250- μl aliquot of the 1 ml ethanol sample was evaporated, re-dissolved in 50 μl of chloroform/heptane/methanol (10:10:1, by vol.) and applied to the column. The majority of urinary steroids were eluted with 14 ml of chloroform/heptane/methanol as eluent; the oestriol/diphenol fraction then was eluted with 4 ml of methanol. The latter fraction was evaporated to dryness under nitrogen, dissolved in ethanol (600 μl) and a 200- μl aliquot taken for derivatisation for GC and GC-MS analysis. GC-MS analysis confirmed that no major steroid contaminants were present in the diphenol fraction and that further elution with methanol gave no other compounds of interest.

Derivatisation

Trimethylsilyl ether (TMS) derivatives were prepared by adding *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)-pyridine (100 μl , 4:1, v/v) to the dry residues obtained by LH-20 chromatography and heating at 60°C for 1 h.

GC and GC-MS

Preliminary identification of major diphenols was based on predetermined reten-

tion indices expressed as methylene units (MU) [25]. They were obtained as the TMS ethers, on a Hewlett-Packard 5710A gas chromatograph equipped for flame ionization and connected to a Shimadzu CR4A integrator for quantitation of areas and for computation of the MU values. Capillary GC was performed on a 30 m SE-30 column using helium as carrier gas with a flow rate of 1 ml/min. Samples (1 μ l TMS ethers) were applied to the column via an all-glass solid injector modified for a syringe-type plunger. Analysis was performed using temperature programming from 197°C to 270°C at 1°C/min. The flash heater was set at 250°C and the detector temperature at 300°C. Quantitation of the compounds was obtained by GC, expressed relative to the amount of cholesterol butyrate (50 ng) co-injected with each derivatised sample.

GC-MS was performed on a Finnigan TSQ-70 mass spectrometer using electron ionisation mode. This was carried out with repetitive scanning over the mass range of 80–800 Da and under the following conditions: temperature of transfer line, 280°C; ionization voltage, 70 eV; and ionization current, 1,200–1,800 μ A. Urinary diphenols were considered identified when GC retention parameters and mass spectra were identical to those of the reference standards.

Control samples

A urinary sample with no detectable levels of equol, daidzein and genistein was spiked with 60 μ g of each of these three standards and used as a control sample in every batch of urine samples assayed. The combined mean recovery of the standards in the spiked samples following enzyme hydrolysis, extraction and purification on LH-20 was 87% (range 82–93%; $n = 11$) as obtained by GC; the three spiked compounds were the only compounds identified by GC.

[³H]oestradiol glucuronide and oestriol were added to each sample at the beginning of the analysis. After enzyme hydrolysis and extraction, the mean percentage recoveries determined by scintillation counting and GC were 80% and 83%, respectively, which was in agreement with the recoveries obtained from the control samples above.

Quantitation of isoflavone levels in soya

Soya flour (5 g) was refluxed in 80% ethanol (100 ml) for 2 h and filtered and evaporated to 5 ml. The extract was diluted to 20 ml with 0.1 M acetate buffer (pH 5) and to this solution 1,400 Fishman units of β -glucosidase (Sigma G-0395) were added and incubated for 48 h at 37°C. The solution was extracted twice with 2 \times volume of diethyl ether and evaporated to dryness under nitrogen. Daidzein, genistein and glycitein levels in the soya flour were quantitated by GC and their identity confirmed by GC-MS.

3. Results

3.1. Isoflavone content of soya

Isoflavone levels in the soya flour were calculated to be 98 mg (genistein), 80 mg (daidzein) and 3 mg (glycitein) per 100 g.

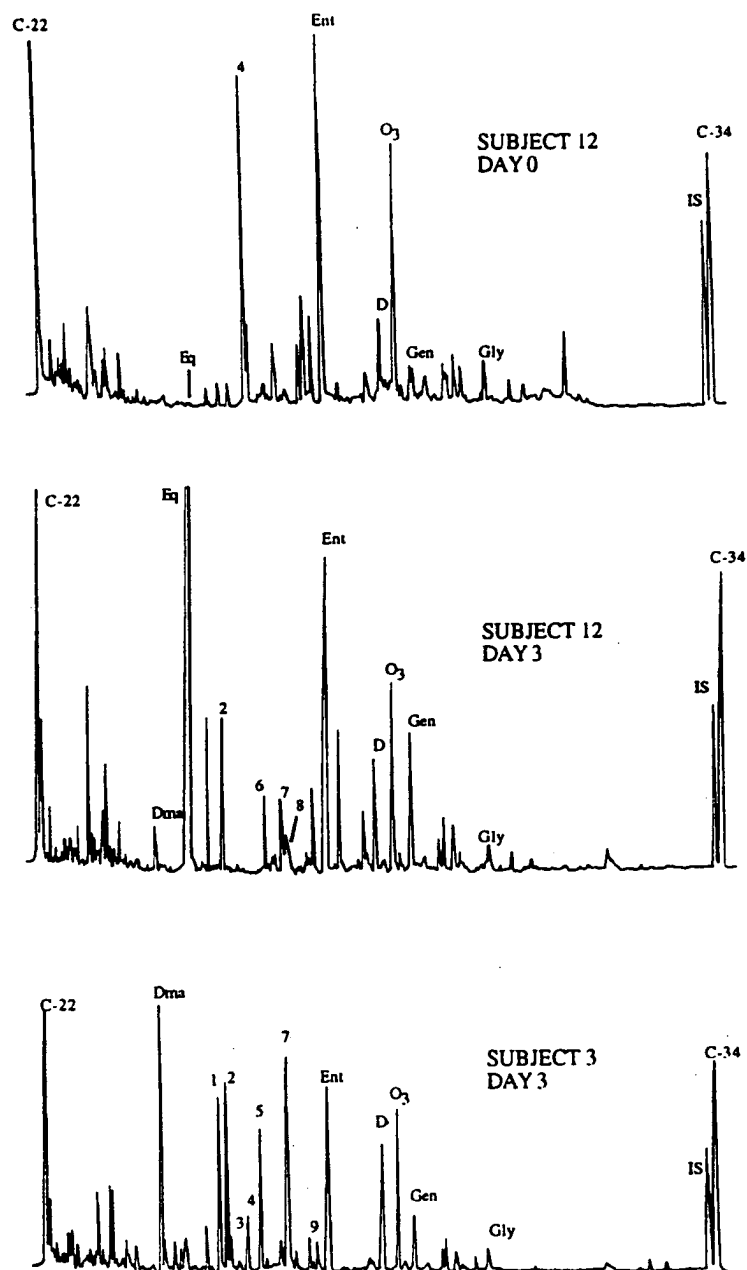


Fig. 1. The urinary diphenol GC profile (TMS ethers) of baseline urine (day 0) of one subject and after (day 3) ingestion of soya flour in two subjects. Cholesterol butyrate (IS); $C_{22}H_{46}$ (C-22); $C_{34}H_{70}$ (C-34); *O*-desmethylanholensin (Dma); equol (Eq); enterolactone (Ent); daidzein (D); oestriol (O₃); genistein (Gen); glycitein (Gly); dehydro-*O*-desmethylanholensin (1); 6'-hydroxy-*O*-desmethylanholensin (2); unknown MU 26.12 (3); unknown MU 26.41 (4); dihydrodaidzein (keto form) (5); tetrahydrodaidzein (6); dihydrodaidzein (enol form) (7); tetrahydrodaidzein (8); dihydrogenistein (9).

3.2. Diphenols in human urine

Two examples of the urinary diphenol profiles obtained by GC following soya challenge are shown in Fig. 1. Levels of diphenols identified in the urine of all 12 volunteers obtained before and after ingestion of soya flour are summarised in Tables 1 and 2.

Daidzein (MU 28.60), genistein (MU 29.15), glycitein (MU 30.35), equol (MU 25.45), *O*-desmethylangolensin (MU 24.85) and enterolactone (MU 27.74), were identified in human urine by GC and GC-MS on the basis of MU values and mass spectral data [18-24].

Five additional diphenols were observed with isoflavonoid-like mass spectra. The first of these novel compounds, (MU 26.03), on the basis of mass spectral data of the TMS ether was characterised as (1-(2',4',6'-trihydroxyphenyl)-2-(4'-hydroxyphenyl-prop-1-one)) (6'-hydroxy-*O*-desmethylangolensin; 6'-OH-*O*-Dma) (M^+ 562, base peak (bp) m/z 369). Two other compounds were observed at MU 27.45 (M^+ 488, bp m/z 298) and MU 25.92 (M^+ 472, bp m/z 281) and were identified by GC-MS as (4',5,7-trihydroxyisoflavonone) (dihydrogenistein) and (1-(2',4',6'-trihydroxyphenyl)-2-(4''-hydroxyphenyl-prop-2-ene-1-one) (dehydro-*O*-desmethylangolensin), respectively. The remaining two novel compounds were

Table 1

Urinary levels ($\mu\text{mol/day}$) of isoflavones, isoflavonoid metabolites and enterolactone in 12 subjects before and 3 days following a soya challenge

Diphenol (MU value)	Days in relation to soya challenge			
	0	3	4	5
Isoflavones				
Daidzein (28.60)	3.81 (1.8-6.6)	14.71 (7.3-25.6)	4.29 (0.1-14.9)	2.08 (0.4-6.3)
Genistein (29.15)	1.15 (0.8-1.96)	8.44 (2.7-19.6)	1.48 (0.2-4.8)	1.44 (0.1-4.8)
Glycitein (30.35)	0.84 (0.02-2.4)	3.59 (0.6-6.4)	0.35 (0.02-0.9)	1.02 (0.1-3.5)
Metabolites				
<i>O</i> -Dma (24.85)	2.34 (ND-0.5)	13.32 (2.3-44.4)	2.67 (0.2-12.7)	1.35 (0.3-3.3)
Equol (25.45)	0.21 (ND-0.7)	9.08 (0.04-61.1)	5.82 (0.02-56.5)	3.55 (0.02-34.7)
6'-OH- <i>O</i> -Dma (26.03)	0.36 (ND-0.8)	7.98 (1.3-24.2)	3.35 (0.3-7.4)	1.28 (ND-3.1)
Dihydrodaidzein (26.65 + 27.11)	1.25 (0.02-3.2)	12.99 (0.5-39.1)	1.09 (0.02-4.0)	1.05 (0.02-2.7)
Enterolactone (27.74)	11.03 (0.9-35.3)	9.29 (7.5-26.9)	7.22 (1.9-22.6)	11.82 (2.5-20.3)

ND, not detected.

Table 2
Total urinary excretion of isoflavones, their metabolites and enterolactone in 12 subjects in a 3-day period immediately following a soya challenge (data expressed as μmol of compound per 72 h total urine volume)

	Subject											
	1	2	3	4	5	6	7	8	9	10	11	12
Sex												
	M	F	F	M	F	F	M	M	F	M	F	M
Isoflavones												
Daidzein	10.07	39.89	12.86	28.52	9.64	40.63	22.34	20.34	11.60	10.35	12.98	24.86
Genistein	3.70	18.17	3.52	17.87	5.70	12.62	23.72	5.55	5.44	15.69	5.00	17.39
Glycitein	2.39	7.00	1.41	7.53	4.19	4.96	4.96	4.47	3.38	0.63	2.74	4.15
Metabolites												
Equol	0.17	0.45	0.50	0.50	0.53	0.83	1.28	7.88	25.10	32.24	49.86	152.32
O-Dma	4.57	3.41	19.36	20.95	31.48	24.97	10.18	58.86	3.41	3.72	16.65	4.07
6'-OH-O-Dma	2.15	5.36	14.55	31.61	23.08	16.88	8.28	12.32	2.81	5.32	0.91	11.92
Dihydrodaidzein	13.31	20.53	28.25	40.19	8.78	32.74	6.44	15.57	7.73	6.95	28.53	8.51
Tetrahydrodaidzein	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.13
Dihydrogenistein	ND	ND	0.84	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dehydro-O-Dma	3.51	ND	5.07	ND	ND	ND	ND	0.51	0.62	ND	ND	ND
Enterolactone	5.77	17.13	27.69	27.25	52.29	40.16	25.81	4.63	2.51	8.75	3.32	73.08

ND, not detected.

observed at MU 26.77 and MU 27.15; (M^+ 474, bp m/z 280) displaying similar fragmentation patterns as exhibited by the two isomers of *cis/trans* (4',4,7-trihydroxyisoflavan) (tetrahydrodaidzein; MU 26.05 and 26.39) which were obtained from daidzein. The complete mass spectral data pertaining to the characterisation of these novel compounds will be presented elsewhere.

GC and GC-MS also revealed the presence of two peaks at MU 26.65 and MU 27.11, which on derivatisation with BSTFA or BSTFA in pyridine and quantitation, appeared to be interconvertible and therefore related. Compound MU 26.65 was identified on the basis of mass spectral data (M^+ 400, bp 192) and by comparison with a reference compound as the keto form of dihydrodaidzein (intermediate O). Compound MU 27.11 was characterised as the enol form of dihydrodaidzein with a molecular ion at m/z 472 (also the bp), a difference of 72 Da, which accounts for the extra TMS ether. The urinary levels of dihydrodaidzein quoted in this study are the combined levels of these two tautomers. Two further compounds with isoflavone-like mass spectra were characterised at MU 26.12 and 26.41, respectively; no attempt was made in this study to identify these two compounds.

A detailed account of data presented in Tables 1–3 follows:

(a) *Isoflavones*: Daidzein, genistein and glycitein were detected in the urine of all 12 subjects prior to the soya challenge; total urinary levels of these three isoflavones ranged between 3.3 and 8.2 $\mu\text{mol/day}$ with daidzein being the predominant isoflavone in each case. Following the soya challenge, each subject showed elevated urinary levels of all three compounds, peaking on day 3 and returning in most cases to basal levels by day 4.

(b) *Isoflavonoid metabolites*: The metabolites equol, O-Dma and 6'-OH-O-Dma were detectable prior to soya challenge in the urines of 9, 10 and 10 of the 12 subjects, respectively. (The limit of detectability for equol by the GC procedure used in this study was established to be 0.02 μmol .) Following the soya challenge, all three metabolites were found in the urine of each subject at elevated levels; the levels peaked on day 3 and then fell progressively over days 4 and 5.

The minor metabolites identified here were detected only in urine collected on day 3 of the study. The tetrahydrodaidzein isomers and dihydrogenistein were identified

Table 3

Comparison of the urinary excretion rates of isoflavones and isoflavonoid metabolites in individuals over the 72 h following 2 consecutive days of soya challenge. Individuals are grouped as either low-equol (less than 8 μmol in 72 h) or high-equol (over 25 μmol in 72 h) producers

	Mean (S.D.) excretion (μmol)	
	Low-equol producer ($n = 8$)	High-equol producers ($n = 4$)
Equol	1.53 (2.60)	64.89 (59.23)
O-Dma	21.72 (17.93)	6.97 (6.47)
6'-OH-O-Dma	14.29 (9.66)	5.25 (4.78)
Daidzein	23.05 (12.43)	14.95 (6.69)
Genistein	11.36 (7.81)	10.88 (6.59)
Male/female	4:4	2:2

in the urine of single subjects only (subjects nos. 12 and 3, respectively); dehydro-O-desmethylangolensin was observed in the urine of four subjects (subjects nos. 1, 3, 8 and 9).

(c) *Enterolactone*: Enterolactone (MU 27.70, M^+ 442, bp m/z 180) was present in relatively high levels in the urine of all 12 volunteers both before and following the soya challenge; enterolactone levels were unaffected by the soya challenge.

(d) *Interrelationship of urinary diphenols*: Individuals were grouped according to their equol excretion rates over the 3 days immediately following the soya challenge, and the relationships between equol and the other diphenols compared and statistically analysed by coefficient of linear regression. These data are summarised in Table 3. A higher rate of excretion of equol appeared to be associated with relatively lower levels of excretion of O-Dma ($r = -0.3$) and 6'OH-O-Dma ($r = -0.18$).

4. Discussion

The data presented here confirm that soya is a rich source of the isoflavones genistein and daidzein and that these undergo varying degrees of intestinal metabolism in humans following ingestion, with both those isoflavones and their metabolites then being absorbed and excreted in the urine.

In soyabeans, various processed soya products and most other legumes studied which are used as human foodstuffs, the principal isoflavones present are daidzein, genistein and glycitein, with low levels of pratensein and prunetin [4,23,26–30]. The isoflavones are present in the plant principally as the glycosides (daidzin, genistin, glycitin) with only approx. 1% present as free aglycones [27,28]. Formononetin and biochanin A which are common in some other legumes mainly used as animal foodstuffs [4], appear to be at low or negligible levels in soya [29]. The isoflavone content of soya varies greatly with soya plant variety as well as with the form of processing used to make the soya flour [27]. In this study, the batch of soya flour used had daidzein and genistein levels of 80 and 98 mg/100 g flour respectively which are at the higher end of the range of levels reported by others [30].

Current knowledge of isoflavone metabolism stems largely from sheep studies. Once ingested, free aglycone forms of genistein, daidzein, biochanin A and formononetin produced by acid or enzymatic hydrolysis of the conjugated isoflavones are available for absorption, appearing in blood and urine as glucuronide conjugates [12,13]; biochanin A and formononetin also may be demethylated by gut bacteria to genistein and daidzein, respectively [31]. Daidzein then is further reduced by gut flora to equol and various other metabolites including angolensin and O-Dma [13,14,31–33]; it has been calculated that about 70% of ingested formononetin in sheep is converted to equol [12]. Genistein is reported to be metabolised largely within the gut by ring cleavage to the non-oestrogenic compound, *para*-ethyl phenol [13,32,33]. Glycitein metabolism has not been described in sheep or other animals.

In humans, the isoflavones formononetin, daidzein and genistein, and the isoflavonoid metabolites equol, dihydrodaidzein, O-Dma and methylequol have been identified in urine [5,6,16,17,19,20,34]. Human faecal flora have been shown to be able to produce equol from soya-rich broth [5] and enzymes capable of carry-

ing out the reduction and deoxygenation reactions in the conversion of daidzein to equol have been identified in human gut flora [35], so it is reasonable to suppose that the metabolism of dietary isoflavones in humans has some common ground with that in sheep.

The relatively low levels of daidzein, genistein, equol and O-Dma found in this study to be present prior to soya challenge in the urine of all 12 subjects on a typically Western omnivorous diet is consistent with the relatively low legume content of that diet. Others [5,6,19,20] similarly report generally low urinary isoflavone levels in non-vegetarian European and North American individuals. However, it is interesting to note that the baseline urinary isoflavone levels found in this study are somewhat higher than in other studies of Western communities [5,6,19,20]. To what extent this apparent difference is due either to methodological factors or to real dietary differences is unclear. None of the 12 test subjects reported knowingly eating any legumes (soya, chick peas, lentils) or legume products (tofu, soymilk, etc.) with relatively high isoflavone content for the week prior to the study, so the urinary isoflavones more than likely derived from leguminous vegetables such as peas, broad beans and peanuts which generally were consumed three to five times weekly in modest amounts by all of the subjects. However, the baseline urinary isoflavone levels in this study were still below those of Japanese maintaining a traditional Japanese diet or of vegetarian subjects in Western communities with significant dietary soya intake on a regular basis [9,20].

The baseline urinary enterolactone levels reported here also were comparatively higher than those reported in other Western surveys of omnivorous individuals [6,20] and may reflect a relatively high consumption of cereal grain in the form of wholemeal bread and breakfast cereals which was reported by most of the 12 subjects. The levels are well below those found in individuals in Finland and the USA with a high cereal grain intake such as in a macrobiotic diet [20], but well above Japanese levels where that country's typical diet is low in cereal grains [9].

The responses of the subjects in this study to a soya challenge produced two noteworthy results. The first was the broad range of metabolites appearing in the urine. A proportion of dietary genistein and daidzein appeared to escape complete metabolic degradation within the intestine, being absorbed as the aglycones which were largely cleared in the urine within 24 h of absorption. However, a proportion of genistein and daidzein underwent more complete intestinal fermentation to yield various intermediate products and end-products which also were readily absorbed and then largely cleared in the urine within 24 h. We have confirmed the presence of equol, dihydrodaidzein and O-Dma as major metabolites of daidzein in humans, as well as describing for the first time four compounds which tentatively have been identified as minor daidzein metabolites, 6'-OH-O-Dma, dehydro-O-Dma and two isomers of tetrahydrodaidzein. The inter-relationships of the various daidzein metabolites cannot be commented on here, but the range of metabolites observed in this study suggests that there may be a number of alternative metabolic pathways; the results of this study also hint at the possibility of an inverse relationship between equol production and that of O-Dma which is in support of alternative metabolic pathways. The metabolic processes in humans associated with genistein are less clear than those with daidzein. *para*-Ethylphenol, the principal genistein metabolite in

sheep, has not been reported in human urine. We are, however, able to identify for the first time the metabolite, dihydrogenistein. Glycitein metabolism has not been described in humans and other animals; in this study, much of the glycitein content of the soya challenge was recovered in the urine as the aglycone, suggesting little if any further metabolism of this particular isoflavone.

The second noteworthy result is the high variability of the metabolic response to isoflavones. In previous studies, others have noted the large variation in urinary equol excretion in response to soya [5,9], an effect confirmed here. In this study, all 12 test subjects showed substantial rises in urinary levels of the three isoflavones (daidzein, genistein, glycitein) following the soya challenge, indicating uniform compliance with the test, as well as absorptive ability of all individuals for isoflavones. Moreover, all 12 subjects showed at least moderate rises in urinary levels of the isoflavonoid metabolites, O-Dma and 6'-OH-O-Dma, indicating the ability in all 12 subjects to metabolise dietary isoflavones. However, the results of this study suggest that while all 12 individuals had the capacity to metabolise dietary isoflavones, there were differences in the particular metabolic pathways used. The reasons for this variability are unclear; this study suggests that gender is an unrelated factor, although genetic factors may be important [9]. In sheep, the overall efficiency of metabolism of dietary isoflavones is influenced by the type of diet fed, although the relative proportions of the different metabolites appears to remain unaltered [31]. This factor has been ascribed to different rates of passage of the different diets through the rumen which is the principal site of detoxification in the sheep [12]. Setchell et al. [5] have cited factors such as the composition of the intestinal microflora, the intestinal transit time and variability in the redox level of the large intestine as possible causes of the variable rates of equol production in humans. Diet is known to influence the level of activity of intestinal bacterial enzymes in humans, including β -glucosidase which is required for hydrolysis of the dietary isoflavones [35]. However, a number of the subjects in this study have been tested repeatedly by the authors over several years and have demonstrated consistent patterns of urinary excretion of the various isoflavonoid metabolites (unpublished), suggesting that the factor responsible for the individual variability may be inherent and less likely to be due to indiscriminant dietary factors.

A number of studies have inferred that dietary isoflavones play an important physiological role in human health [5–9]. Isoflavones exhibit an affinity for oestrogen receptor sites and may therefore be considered to function as anti-oestrogens through competitive inhibition [2,36,37]. The complexing of the isoflavone with the cytosol oestrogen receptor is thought to inhibit the biosynthetic process associated with the receptor as well as inhibiting the replacement of the receptor leading to a diminished concentration of the cytosol receptors. Genistein, daidzein and equol have relatively strong affinities for oestrogen receptors, with O-Dma showing much weaker binding affinity and glycitein appearing to be non-oestrogenic [2,24,36,37]. Notwithstanding the weaker affinity of the isoflavones and their metabolites for oestrogen receptors relative to oestradiol [2,36,37], the finding that all individuals in this study challenged with soya showed urinary isoflavonoid metabolite levels greatly in excess of the classical oestrogens [38], points to a potentially significant *in vivo* effect in those individuals on endogenous oestrogen metabolism and function.

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